present application and provide section headings where appropriate. A marked up version of the amended sections of the specification showing the amendments made herein is attached hereto as Exhibit A. In Exhibit A, the addition of matter is indicated by bold text in view of the fact that the section headings are intended to be underlined in the text of the specification. The amendments to the specification do not introduce new matter as defined in 35 U.S.C. § 132.

Claims 1-20 have been canceled without prejudice to Applicants' right to prosecute the subject matter of the canceled claims in related applications. New claims 53-59 have been added to more particularly point out and distinctly claim that which Applicant regards as the invention. The new claims are fully supported by the instant specification (see, *e.g.*, page 1, lines 10-17, page 4, line 31 to page 5, line 7, page 7, lines 1-46, page 8, lines 38-42, and the examples presented at pages 11-24), and do not represent new subject matter. After entry of the amendments made herein, claims 53-59 will be pending in the instant application. A copy of the pending claims is attached hereto as Exibit B.

Claims 53-59 are drawn to methods of treating an asthmatic disorder, comprising administering an 8F4[1] inhibitory molecule, such as a monoclonal antibody that recognizes the human 8F4 polypeptide or an 8F4 polypeptide, to an individual in need of such treatment. Applicant submits herewith a Declaration of Richard Kroczek Under 37 C.F.R. § 1.132 ("Kroczek Declaration"; Exhibit C), presenting data that corroborates the teachings of the instant application relating to the presently claimed invention.

The Kroczek Declaration describes, first, a study conducted by Gonzalo *et al.* (2001, "ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses," Nat. Immunol. 2(7):597-604) demonstrating that administration of ICOS-inhibitory compounds, including an antibody that recognizes ICOS and a soluble ICOS polypeptide, results in abrogation of symptoms of asthma in an art-accepted mouse model of the disease (see ¶¶ 6 to 11 of the Kroczek Declaration). In addition to the data generated in an art-accepted model, the Kroczek Declaration, in paragraphs 12 to 14, further describes

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[1] It is noted that since the original filing date of the instant specification, the 8F4 polypeptide has come to be referred to in the literature as "ICOS" (Inducible T cell CO-Stimulator). As such, throughout this Amendment, Applicant will generally refer to 8F4 as "ICOS."

experiments demonstrating that in humans ICOS-expressing cells are associated with lung inflammation resulting in asthma. The Kroczek Declaration, therefore, presents mouse and human data corroborating the teachings of the present application that successful *in vivo* amelioration of asthma symptoms can be achieved by administration of antibodies that recognize the human ICOS gene product or the ICOS gene product itself.

Entry of the amendments and remarks made herein is respectfully requested.

Respectfully submitted,

Date October 4, 2001

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)

By: Muna Abu-Shaar

Muna Abu-Shaar
Limited Recognition Under 37 C.F.R. § 10.9(b)
Copy of Certificate Enclosed

PENNIE & EDMONDS LLP
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New York, New York 10036-2711
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Enclosures

Exhibit A

Marked up Version of Amended Paragraphs in Specification

Marked up version of amended title:

[Costimulating polypeptide of T cells, monoclonal antibodies, and the preparation and use thereof] **Methods For Treatment of Asthmatic Disorders**

New paragraph and subsequent paragraph heading added after title:

Cross Reference To Related Applications

This application claims priority to U.S. Patent Application No. 09/509,283, filed August 11, 2000, PCT Application PCT/DE98/02896, filed September 23, 1998, and German Applications DE 19821060.4, filed May 11, 1998, and DE 19741929.1, filed September 23, 1997, each of which is incorporated herein by reference in its entirety.

Background of the Invention

New paragraph heading added after line 36 on page 3:

Summary of the Invention

Marked up copy of replacement paragraph heading on page 8, lines 8-9:

[The figures serve to illustrate the invention]

Brief Description of the Drawings

Exhibit B

Claims as Pending Following Entry of Amendments Made Herein

53. (New) A method for treating an asthmatic disorder, comprising: administering to an individual in need of treatment an 8F4 inhibitory molecule selected from the group consisting of an 8F4 polypeptide and a monoclonal antibody that recognizes a human 8F4 polypeptide, wherein said 8F4 polypeptide:

a) is an inducible T cell costimulatory molecule;

b) occurs on two-signal-activated human T lymphocytes;

c) exhibits a molecular weight of about 55 to 60 kilodaltons as determined by non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); and

d) is a dimer of two peptide chains exhibiting molecular weights of about 27 kilodaltons and 29 kilodaltons, as measured by reducing SDS-PAGE,

in an amount sufficient to ameliorate a symptom of the asthmatic disorder, such that the asthmatic disorder is treated.

54. (New) The method of claim 53, wherein the 8F4 inhibitory molecule is a monoclonal antibody that recognizes a human 8F4 polypeptide.

55. (New) The method of Claim 54, wherein the monoclonal antibody recognizes the human 8F4 polypeptide of about 55 kilodaltons to 60 kilodaltons, as determined by non-reducing SDS-PAGE.

56. (New) The method of Claim 54, wherein the monoclonal antibody recognizes the peptide chain of about 27 kilodaltons, as determined by reducing SDS-PAGE.

57. (New) The method of Claim 54, wherein the monoclonal antibody recognizes the peptide chain of about 29 kilodaltons, as determined by reducing SDS-PAGE.

58. (New) The method of Claim 54, wherein the monoclonal antibody recognizes a human 8F4 polypeptide present on activated human $CD4^+$ T lymphocytes and activated human $CD8^+$ T lymphocytes.

59. (New) The method of claim 53, wherein the 8F4 inhibitory molecule is an 8F4 polypeptide.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Kroczek

Application No.: To be assigned | Group Art Unit: To be assigned

Filed: Herewith | Examiner: To be assigned

For: METHODS FOR TREATMENT OF ASTHMATIC DISORDERS (as amended) | Attorney Docket No.: 7853-240

DECLARATION OF RICHARD KROCZEK UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, RICHARD KROCZEK do declare and state:

1. I am the inventor of the invention described and claimed in the above-identified patent application.

2. I presently hold the position of Professor of Molecular Immunology at the Robert Koch Institute, Berlin, Germany, the assignee of the above-identified patent application. My *curriculum vitae* is attached hereto as Exhibit 1.

3. I have read and am familiar with the instant application.

4. The invention claimed in the present application is directed to methods of treating asthmatic disorders comprising administering monoclonal antibodies directed against a human polypeptide, referred to in the present application as the "8F4 polypeptide." Since the original filing date of the specification of PCT Application PCT/DE98/02896, to which the present application claims priority, the 8F4 polypeptide has come to be referred to in the literature as "ICOS" (Inducible T cell Co-Stimulator). A polypeptide corresponding to an 8F4 polypeptide has also been referred to in the literature as "H4."

5. Described in paragraphs 6 to 11 below is a study by Gonzalo *et al.* (2001, "ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses," Nat. Immunol. 2(7):597-604, attached hereto as Exhibit 2)("Gonzalo"), of which I am a co-author, that demonstrates that administration of ICOS-inhibitory compounds, such an antibody that recognizes ICOS and an ICOS polypeptide, in an accepted experimental model of human asthma, result in abrogation of symptoms of the disease. Further described in paragraphs 12 to 14 below are experiments done by me or by others under my supervision that demonstrate that ICOS-expressing cells are associated with lung inflammation resulting in asthma in humans. These experiments corroborate the teaching provided in the present application by indicating that successful *in vivo* amelioration of asthma symptoms can be achieved by administration of antibodies that recognize the human ICOS polypeptide or the ICOS polypeptide itself.

INHIBITION OF ASTHMA SYMPTOMS BY ICOS-INHIBITORY MOLECULES IN A MOUSE MODEL OF ASTHMA

6. The study reported by Gonzalo provides evidence of the critical role of ICOS in the pathology of asthma. The experiments described by Gonzalo utilize an art-recognized mouse model for asthma, DO11.10 (Wills-Karp, 2000, Immunopharmacology 48:263-268, attached hereto as Exhibit 3). DO11.10 transgenic mice express a T cell receptor specific for the OVA antigen (amino acid residues 323-339 of chicken ovalbumin). Briefly, upon exposure to particular antigens, DO11.10 mice exhibit symptoms (*e.g.*, lung mucosal inflammation) that are characteristic of asthma in humans. The severity of the symptoms in such treated mice was notably reduced by administration of ICOS inhibitory molecules to the mice.

7. More specifically, exposure of DO11.10 mice to the OVA antigen results in the production of a variety of inflammation-promoting cytokines in the bronchioalveolar fluid (BAL) of the mice. In contrast, as described in the Section at page 600 entitled "ICOS- and CD28-mediated cytokine regulation" and the accompanying Figure 7, administration of a monoclonal antibody that recognizes ICOS, 12A8, prior to exposure of the mice to the OVA antigen reduces the levels of inflammatory cytokines in the BAL of the mice relative to untreated DO11.10 mice. Similarly, administration of a soluble form of ICOS polypeptide, ICOS-Ig, prior to exposure of the mice to the OVA antigen reduces the levels of inflammatory cytokines in the BAL of the mice relative to untreated DO11.10 mice. Thus, administration of a monoclonal antibody that recognizes ICOS or an ICOS polypeptide reduces a hallmark of asthma.

8. Another hallmark of asthma in humans is accumulation of lymphocytes and eosinophils in the BAL upon exposure to antigenic stimulus. After persistent exposure to OVA, DO11.10 mice exhibit such accumulation. As described in the Section on page 599 entitled "Regulation of mucosal inflammation by ICOS" and Figure 5, the administration of 12A8 antibody DO11.10 mice before each exposure to OVA reduces lymphocyte and eosinophil accumulation by 50 and 70%, respectively. The authors of Gonzalo further note, also as shown in Figure 5, that comparable suppression of lymphocyte and eosinophil accumulation observed upon administering ICOS-Ig. Again, this represents a reduction in a hallmark of asthma.

9. In summary, the experimental results presented in Gonzalo demonstrate that administration of ICOS inhibitory compounds, such as anti-ICOS antibodies or ICOS proteins, can ameliorate symptoms of asthma.

ICOS IN HUMAN ASTHMA

10. The results presented hereinbelow demonstrate that ICOS is, indeed, involved in human lung inflammation such as that seen in asthma. This human data further evidences that administration of ICOS inhibitory compounds to humans will mirror the effects of administering such compounds to the art accepted model of human asthma, and accordingly, that ICOS inhibitory compounds will be useful in treating human asthmatic disorders.

11. Airway hyperresponsiveness and pulmonary inflammation are the two central hallmarks of human allergic asthma. The inflammatory process is initiated and maintained by T cells. The crucial role of certain co-stimulatory pathways in allergic asthma has been demonstrated in mice and several studies in the human (Exhibits 4-5).

12. To assess the participation of the ICOS molecule in the pathogenesis of human asthma, individuals with mild asthma were exposed to allergen. The expression of ICOS on T cells present in the bronchoalveolar lavage (BAL) fluid collected 42 hours after segmental allergen provocation was analyzed by flow cytometry, as described in Exhibit 6. These data, described in Exhibit 7, were correlated with immunohistological studies on ICOS expression in bronchial biopsies taken at the time the performed BAL was performed.

13. The data clearly demonstrate that a very substantial proportion of the infiltrating T cells found in the submucosa and in the epithelium bear significant levels of ICOS. These ICOS-positive T cells transmigrate into the bronchial space on allergen exposure.

14. The finding that ICOS is involved in human lung inflammation validates the results obtained by Gonzalo demonstrating the inhibition of hallmarks of asthma by ICOS inhibitory molecules in a mouse model of asthma, and signifies that ICOS inhibitory compounds will have similar effects on human asthma. Thus, taken together, the human data presented herein and the mouse model data shown by Gonzalo strongly indicate that: a) ICOS participates in the pathogenesis of allergic asthma in humans; and b) amelioration of asthma symptoms in humans can be achieved by administration of antibodies that recognize the ICOS polypeptide or by administration of ICOS polypeptides.

15. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: October 4, 2001 R. Kroczek

Richard Kroczek

Attachments:

Exhibit 1: *Curriculum Vitae* of Richard Kroczek

Exhibit 2: Gonzalo *et al.*, 2001, "ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses," Nat. Immunol. 2(7):597-604.

Exhibit 3: Wills-Karp, 2000, Immunopharmacology 48:263-268.

Exhibit 4: Keane-Myers *et al.*, 1998, "Development of murine allergic asthma is dependent upon B7-2 costimulation," J. Immunol. 160:1036-1043.

Exhibit 5: Mathur *et al.*, 1999, "CD28 interaction with either CD80 or CD86 are sufficient to induce allergic airway inflammation in mice," Am. J. Respir. Cell. Mol. Biol. 21:498-509.

Exhibit 6: Materials and Methods for Human Asthma Analysis

Exhibit 7: Results for Human Asthma Analysis

CURRICULUM VITAE

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Personal Information: born November 3, 1952 in Orlau
Nationality: German

Education and Training:

1964 - 1973	Attending the Hans-Leinberger-Gymnasium in Landshut, Germany; graduated first in the class of 1973
1973 - 1976	Pre-clinical studies at the University of Kiel
1976 - 1977	Medical School, University of Bonn
1977 - 1978	Westminster Hospital Medical School, London, supported by a grant from the Deutscher Akademischer Austauschdienst
1978 - 1981	Continuation of clinical studies at the University of Bonn
1981	Final Medical Exam ("Staatsexamen"); Doctoral thesis; Medical License
1981 - 1983	Residency in Pediatrics at the Munich University Children's Hospital
1983	American Medical Exam (VQE)
1984 - 1986	Postdoctoral Fellow in Immunology with Dr. Ethan Shevach in the Laboratory of Allergy and Infectious Diseases, NIH, USA. Supported by a grant from the Deutsche Forschungsgemeinschaft. Research topics: Role of Thy-1 in T-cell activation, action of cyclosporin A
1986	Research fellow of the Fogarty Foundation

1986 - 1987	Postdoctoral fellow at the Max-Planck-Institute for Immunobiology in Freiburg

Employment:

1987 - 1992	Head of a research group at the Max-Planck-Society Research Unit for Immunology in Erlangen, Germany
1990	Habilitation at the University of Erlangen; faculty member of the university
1997	Professor, University of Erlangen
1993 -	Head, Molecular Immunology, Robert Koch-Institute, Berlin
1999	Offered chair in immunology at the Free University of Berlin (not accepted)

Current research: Molecular mechanisms of early T cell activation, T cell/B cell cooperation, T cell/monocyte cooperation, T cell/dendritic cell cooperation focus on the function of CD40 Ligand, ATAC and ICOS molecules in vitro and in vivo

Professional and scientific activities:

Member of the German Society for Immunology.

Reviewer for various scientific journals (European Journal of Immunology, Journal of Immunology, European Journal of Biochemistry, Blood, Journal of Clinical Investigation, Nature Medicine).

Reviewer for various scientific societies and funding agencies.

Honors:

Science prize of the SmithKline Beecham Foundation 1999.

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Nature Immunology 2 (2001) 597-604

ICOS is critical for T helper cell–mediated lung mucosal inflammatory responses

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We examined the requirement for and cooperation between CD28 and inducible costimulator (ICOS) in effective T helper (T_H) cell responses *in vivo*. We found that both CD28 and ICOS were critical in determining the outcome of an immune response; cytolytic T lymphocyte–associated antigen 4–immunoglobulin (CTLA-4–Ig), ICOS-Ig and/or a neutralizing ICOS monoclonal antibody attenuated T cell expansion, T_H2 cytokine production and eosinophilic inflammation. CD28-dependent signaling was essential during priming, whereas ICOS-B7RP-1 regulated T_H effector responses, and the up-regulation of chemokine receptors that determine T cell migration. Our data suggests a scenario whereby both molecules regulate the outcome of the immune response but play separate key roles: CD28 primes T cells and ICOS regulates effector responses.

Optimal T cell expansion is regulated by signals delivered through the T cell receptor (TCR) and a number of costimulatory molecules[1,2]. CD28 ligation on antigen-inexperienced precursor T helper (T_HP) cells by its counter-receptors B7-1 and B7-2, which are expressed on dendritic cells, play a crucial role in initial T cell priming, interleukin 2 (IL-2) production and cycle cell progression[3–5]. Recently primed T_HP cells migrate to the T cell zone of the B cell follicle where they encounter antigen- specific B cells that have also migrated to this area[6]. T cells deliver, primarily through CD40, a signal for B cell expansion. Primed T cells in turn receive additional signals from B cells that regulate T_H function. CD28-mediated T cell expansion is opposed by cytolytic T lymphocyte–associated antigen 4 (CTLA-4)[7], which also binds both B7-1 and B7-2; its function is to attenuate the T cell expansion and cytokine production of recently activated T cells. The importance of CTLA-4–mediated suppression is illustrated in gene-deficient mice, which exhibit autoimmune disease characterized by marked T cell expansion and death within 3–4 weeks[4].

The inducible costimulatory molecule (ICOS)[9] is the third member of the CD28 superfamily and, like CTLA-4, is expressed on antigen-primed T cells[9,10]. ICOS binds its own B7 family member, B7RP-1[11] or B7h[12], on B cells and macrophages[11]. ICOS can induce CD28-independent T cell expansion and selective cytokine production[9,11]. Similarly, signaling though CD28 provides a signal for T_H2 cytokine production *in vitro*[13]. It has been proposed that T_H2 responses are more dependent on CD28-mediated costimulation than T_H1 responses, although this remains somewhat controversial[3]. Data from ICOS-deficient mice have shown that this molecule plays a critical role in T–B cell interactions, is essential for germinal center formation and humoral immune responses and delivers a key signal for IL-4, but not interferon-γ (IFN-γ), production[14,15]. However, the contribution of these different costimulatory effects in regulating T cell function during inflammatory processes remains unknown.

Effective immune responses require not only the appropriate costimulatory signals necessary for optimal cytokine production but also the coordinated migration of T cells into the lymph nodes and target tissues, a process that is critically regulated by chemokines and their receptors[17]. T_H effector cells can be divided not only on the basis of their ability to produce different cytokines but also on the differential expression of chemokine receptors. T_H1 cells express the chemokine receptors CXCR3 and CCR5, whereas T_H2 cells express CCR3, CCR4 and CCR8[18,20]. This differential chemokine receptor expression may play an important role in determining whether T_H1 or T_H2 cells accumulate at sites of delayed-type hypersensitivity or allergic inflammation. The relationship, however, between costimulatory signals and chemokine receptor–mediated T cell migration remains largely unexplored.

We have investigated the role of ICOS and CD28 in regulating T_H2-mediated mucosal inflammatory responses. Our data shows that CD28 is critical during priming but does not contribute to recall responses once effector T_H2 cells have been generated. In contrast, ICOS plays the predominant role in regulating T_H2 effector cell activation. ICOS-mediated signaling contributes to the inflammatory response not only through the regulation of IL-4 but by providing a signal for up-regulation of the chemokine receptors CCR3, CCR4 and CCR8. The findings reported here indicate that strategies aimed at inhibition of ICOS may represent a therapeutic target during allergic inflammatory responses and other disorders characterized by inappropriate T cell activation.

Results

Generation of neutralizing mAbs to ICOS

Monoclonal antibodies (mAbs) to ICOS, 12A8 and 1C10, were used to stain ICOS-transfected Jurkat cells (ICOS$^+$ cells), which were assessed by flow cytometry; they did not stain untransfected Jurkat cells (**Fig. 1a**). Activated, but not resting, CD4$^+$ T cells were also stained by 12A8

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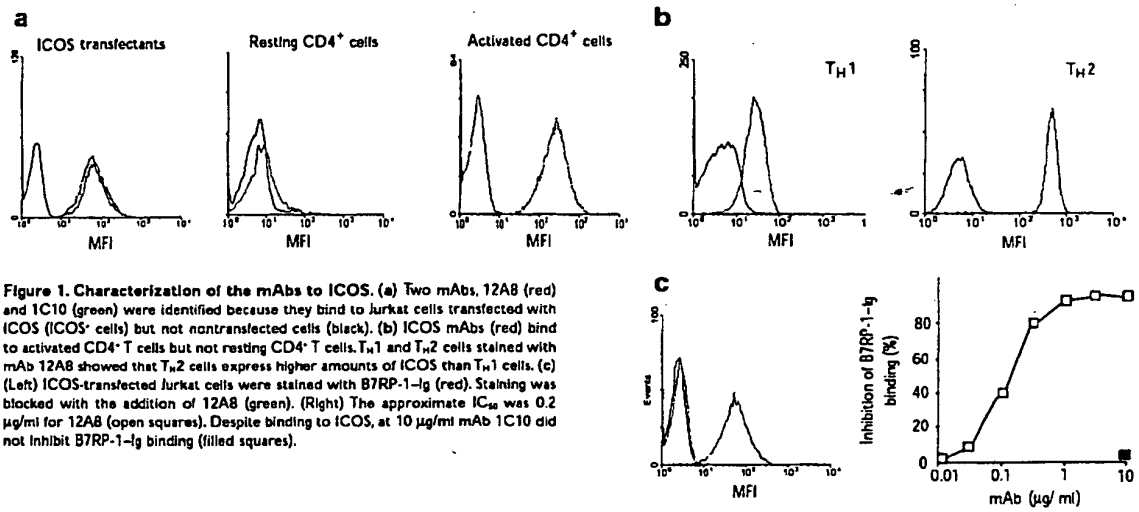

Figure 1. Characterization of the mAbs to ICOS. (**a**) Two mAbs, 12A8 (red) and 1C10 (green) were identified because they bind to Jurkat cells transfected with ICOS (ICOS$^+$ cells) but not nontransfected cells (black). (**b**) ICOS mAbs (red) bind to activated CD4$^+$ T cells but not resting CD4$^+$ T cells. T_H1 and T_H2 cells stained with mAb 12A8 showed that T_H2 cells express higher amounts of ICOS than T_H1 cells. (**c**) (Left) ICOS-transfected Jurkat cells were stained with B7RP-1–Ig (red). Staining was blocked with the addition of 12A8 (green). (Right) The approximate IC_{50} was 0.2 μg/ml for 12A8 (open squares). Despite binding to ICOS, at 10 μg/ml mAb 1C10 did not inhibit B7RP-1–Ig binding (filled squares).

(**Fig. 1a**). When cells were cultured in the presence of IL-4, ICOS expression was enhanced, whereas repetitive antigenic stimulation in the presence of IL-12 resulted in a down-regulation of ICOS expression (**Fig. 1b**). The ability of these mAbs to prevent binding of the ICOS ligand B7RP-1 was also assessed. We found that 12A8 inhibited B7RP1-immunoglobulin (Ig) binding with an IC_{50} of ~0.2 μg/ml (**Fig. 1c**). In contrast, 1C10, which stained ICOS-expressing cells, was unable to block B7RP1-Ig binding (**Fig. 1c**). Neither 12A8 nor 1C10 bound to CD28 transfectants or inhibited B7-1–Ig binding to activated T cells (data not shown). Thus, 12A8 recognized an epitope on ICOS that was common to that used for ligand binding. In contrast, although 1C10 recognized ICOS, it bound to a site that was distinct from B7RP-1.

Cytokine production after ICOS neutralization

We next assessed the role of ICOS in T_H cell function. We determined cytokine production by TCR-transgenic T cells after stimulation with the ovalbumin peptide OVA(323–339), referred to hereafter as OVA, *in vitro* or immunization with keyhole limpet hemocyanine (KLH) *ex vivo* + *in vivo* treatment with mAbs to ICOS. We found that mAb 12A8 inhibited IL-4, IL-10 production and, to a lesser extent, IL-5 production by CD4$^+$ T cells (**Fig. 2a**). In contrast, the production of the T_H1-type IFN-γ was increased after treatment with 12A8 (**Fig. 2a**). *In vivo* treatment with neutralizing 12A8 also inhibited IL-4 and IL-10 production (**Fig. 2b**). The mAb 1C10, which was unable to block B7RP-1–Ig binding, did not alter cytokine release in response to OVA or KLH; these results were not different those obtained with the use of control rat Ig. These data indicate that signaling through ICOS delivers a signal for immune deviation that facilitates T_H2 cytokine expression and reduces T_H1 cytokine production.

Expression of CD28-B7 family members in lungs

CD28, CTLA-4 and ICOS mRNA expression was measured by Taqman analysis in lung tissue isolated at different time-points during lung allergic inflammation. The inflammatory response to OVA consisted of

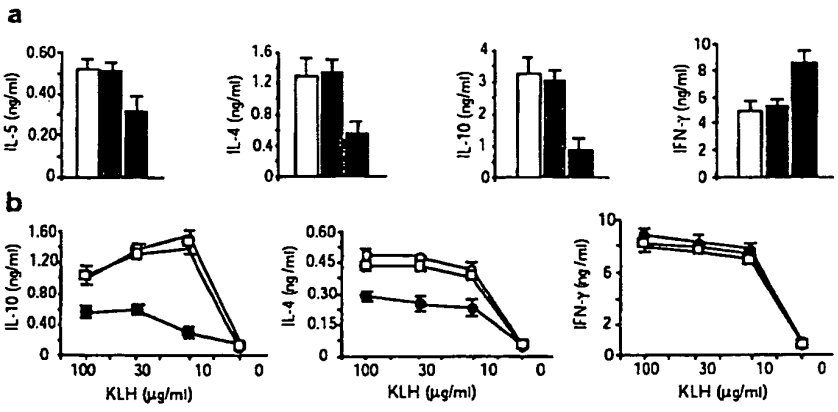

Figure 2. Antigen-induced cytokine production after ICOS neutralization. (**a**) Cytokines were measured in the supernatant of OVA-specific TCR-transgenic spleen CD4$^+$ T cell cultures stimulated with OVA peptide (0.3 μg/ml). The neutralizing anti-ICOS mAb 12A8 (black bars) or the nonneutralizing anti-ICOS mAb 1C10 (shaded bars) were added to cultures. Rat Ig (open bars) was used as a control. (**b**) Cytokines in the supernatant of draining lymph node cells collected from KLH-immunized mice after *in vivo* treatment with 12A8 (closed circles) or 1C10 (open circles) were measured. Rat Ig (open squares) was used as a control.

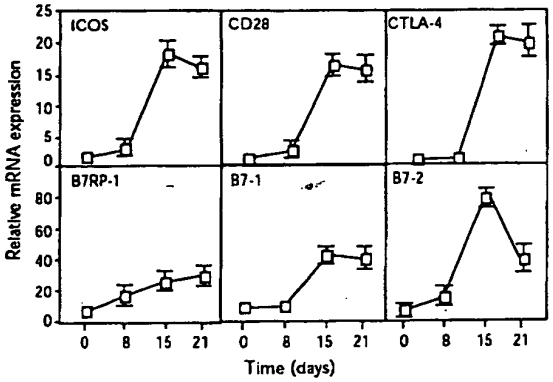

Figure 3. ICOS expression during allergic lung inflammation. Lungs from mice subjected to OVA treatment, 3 h after antigen challenge, on days 0, 8, 15 or 21 were analyzed. Each square represents mean mRNA expression of CD28 and B7 family members from five mice at the time-points indicated.

an accumulation of macrophages in the lung (interstitium and airway lumen) that was maximal in the early stages (3 h after OVA challenge on day 15); the accumulation of eosinophils and lymphocytes reached a plateau in the late stages of the response (3 h after OVA challenge on day 21). CD28, CTLA-4 and ICOS were expressed in low amounts in the lungs on day 0 and day 8 (**Fig. 3**), However CD28, CTLA-4 and ICOS expression were up-regulated by day 15 of OVA treatment and expression was still maintained by day 21. B7RP-1 was also expressed in the lungs and was maximal by day 21. Similarly, B7-1 and B7-2 mRNA expression was up-regulated by four- and eightfold, respectively, during the course of the inflammatory response (**Fig. 3**).

To determine the location of ICOS protein expression, lung sections were stained with mAbs to ICOS. No ICOS expression was detected in the lung of PBS-treated mice, but increased expression was observed in the infiltrating parenchymal T cells of OVA-treated animals (**Fig. 4a,b**). In addition, lymph nodes from mice immunized with KLH were stained with the mAb 12A8 (**Fig. 4c–e**). The sections showed that ICOS$^+$ cells were present within the B cell follicle after immunization (**Fig. 4c**). The relationship between ICOS$^+$ cells and germinal center formation was also determined. By day 4 after immunization with KLH, ICOS$^+$ cells could be detected in close proximity to B cells with the early formation of peanut agglutinin–positive (PNA$^+$) germinal centers (**Fig. 4d**). By day 7, distinct PNA$^+$ cells surrounded by ICOS$^+$ cells could be seen in the lymph nodes (**Fig. 4e**).

Regulation of mucosal inflammation by ICOS

Leukocyte cell numbers in the lungs of OVA-treated mice were determined 3 h after OVA challenge on day 21. After treatment with mAb 12A8, lymphocyte and eosinophil numbers in the broncholaveolar lavage (BAL) fluid of OVA-treated mice were reduced by 50% and 70%, respectively (**Fig. 5a,b**). Comparable suppression was observed with ICOS-Ig. CTLA-4–Ig induced an almost complete reduction in BAL lymphocyte and eosinophil accumulation. As a control, mAb 1C10 was used. The results obtained with 1C10 did not differ from those obtained with experiments with rat Ig–treated animals or with human Ig, which was used as a control for CTLA-4–Ig and ICOS-Ig. For clarity, only data obtained with 1C10 are shown. These observations were supported by the examination of lung sections, which were assigned a score based on the extent and size of peribronchiolar infiltrates. Animals treated with OVA + the control mAb 1C10 had a mean score of 4.7±0.4; administration of 12A8 during OVA treatment decreased the mean score to 2.8±1.1.

To determine whether OVA-induced inflammatory cell accumulation in the lung was prevented rather than delayed by administration of mAb 12A8, the BAL fluid of OVA-treated mice was collected at different time-point on day 21 and eosinophil numbers evaluated. After treatment with mAb 12A8, eosinophil accumulation was reduced at all time-points investigated (**Fig. 5c**). Similarly, ICOS blockade with 12A8 or ICOS-Ig or CD28 blockade with CTLA-4–Ig abrogated antigen-induced airway hyperresponsiveness (AHR) (**Fig. 5d**).

Regulation of priming and effector responses

Thus far, our data show that CD28 and ICOS both contribute to T cell–mediated inflammatory responses. We next dissected the contribution of ICOS and CD28 during either priming or effector responses.

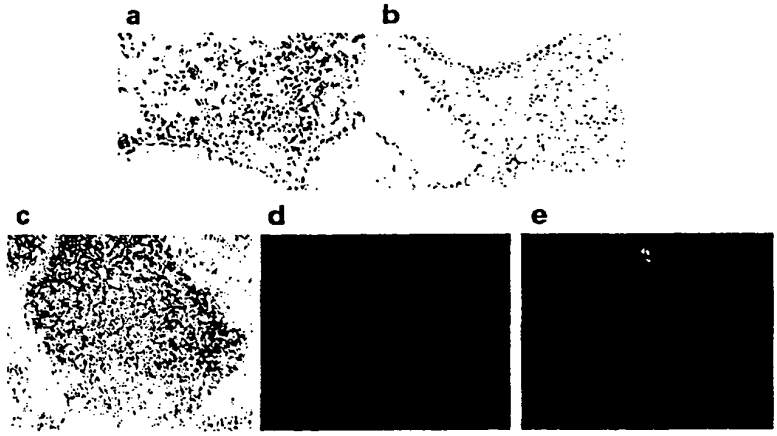

Figure 4. ICOS expression in the lung and peribronchiolar lymph nodes during allergic lung inflammation. (**a**) Sections were prepared from mice on day 21 of OVA treatment and were stained with mAb 12A8. ICOS staining in the lung was indicated by a brown reaction product on a blue counterstain. (**b**) ICOS expression was undetectable in lungs from PBS-treated controls (**c–e**) Lymph node sections from KLH-immunized mice were stained with horseradish peroxidase (HRP)–12A8 (brown) and alkaline phosphatase–B220 (blue, to reveal B cells) and showed ICOS$^+$ cells within the B cell follicle on day 7 after immunization (**c**). Immunofluorescence staining on day 4 after immunization showed ICOS$^+$ cells (red) associated with B220$^+$ B cells (green) within the follicle and the formation of a germinal center, as revealed by PNA$^+$ cells (blue) (**d**). Day 7 after immunization showed a germinal center in the lymph node (blue), with B cells stained in green and ICOS$^+$ cells in red (**e**). Original magnification: ×40.

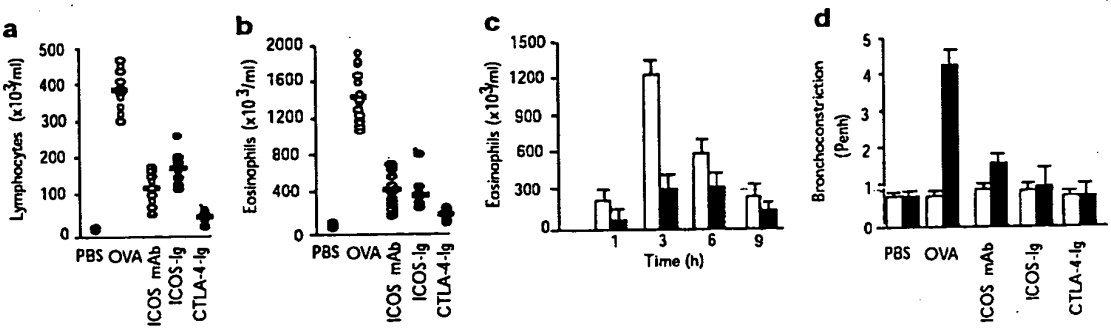

Figure 5. OVA-induced BAL leukocyte accumulation and AHR after ICOS neutralization during allergic lung inflammation. Neutralizing mAb 12A8 (30 μg/mouse, shaded circles), control mAb 1C10 (30 μg/mouse, open circles), ICOS-Ig (100 μg/mouse, blue circles) or CTLA-4–Ig (100 μg/mouse, red circles) were administered before each OVA challenge. Mice exposed to PBS are shown for comparison (filled circles). Accumulation of (a) lymphocytes and (b) eosinophils in the BAL fluid. Each circle represents an individual animal; bars represent the mean for each group. (c) Total number of eosinophils recovered from the BAL fluid of OVA + 12A8–treated mice (filled bars) or OVA + 1C10–treated controls (open bars). Data are the mean±s.e.m. eosinophil numbers (n=10). (d) AHR is shown as are the mean±s.e.m. Penh before (open bars) and after (filled bars) methacholine (MCh) challenge (n=10).

Anti-ICOS (mAb 12A8), ICOS-Ig or CTLA-4–Ig were administered either during priming (on day 0 and 8) or during the efferent response (on day 21 alone). OVA-induced lymphocyte and eosinophil accumulation in the airways (as assessed by analysis of the BAL fluid) were not affected when 12A8 or ICOS-Ig was administered on days 0 and 8. However, when 12A8 or ICOS-Ig were administered on day 21, a 50% reduction in the total number of lymphocytes and eosinophils in the lung was observed (**Fig. 6**). This indicated that signals delivered by ICOS were important for the activation or recruitment of effector T cells. Changes in the composition of the BAL fluid were associated with corresponding changes in the lung interstitum, as assessed by histology (data not shown). Similarly, CTLA-4–Ig inhibited AHR when administered during priming, whereas ICOS mAb 12A8 or ICOS-Ig inhibited AHR when administered on day 21, when CD28 blockade was not effective (**Fig. 6**)

ICOS- and CD28-mediated cytokine regulation

To determine whether the differential regulation of mucosal inflammation by ICOS and CD28 could be explained by differentially regulating T cell activation, we measured cytokine production in the BAL fluid after challenge. Treatments, from day 0–21, with both CTLA-4–Ig and mAbs to ICOS suppressed production of IL-4, IL-5, IL-10 and IL-13. In contrast, IL-12 production was not inhibited by treatment with CTLA-4–Ig, ICOS-Ig or mAbs to ICOS. In response to OVA, no IFN-γ production was detected in the BAL fluid, which indicated that aerochallenge had induced a T_H2 effector response. In addition, treatment with anti-ICOS therapies, which attenuate T_H2 responses, did not result in immune deviation, that is, IFN-γ concentrations were not augmented as a consequence of ICOS blockade (**Fig. 7**).

Animals were treated with CTLA-4–Ig, ICOS-Ig or mAbs to ICOS either during priming or before the final aeroallergen challenge only, when T_H2 effector cells had been generated. When administered on days 0 and 8, CTLA-4–Ig reduced T_H2 cytokine production; when administered on day 21, it did not regulate IL-4, IL-10 or IL-13 production, although a 50% reduction in IL-5 was observed. In contrast, ICOS blockade on day 21 effectively reduced the production of IL-4, IL-5, IL-10 and IL-13. We also found that ICOS blockade during priming had no effect on IL-4, IL-5 or IL-13 production, although, when administered on days 0 and 8, effective suppression of IL-10 production was observed (**Fig. 7**).

ICOS-dependent chemokine receptor expression

The contribution of ICOS in the effector phase of the lung mucosal inflammatory response may be, at least in part, explained by a failure to

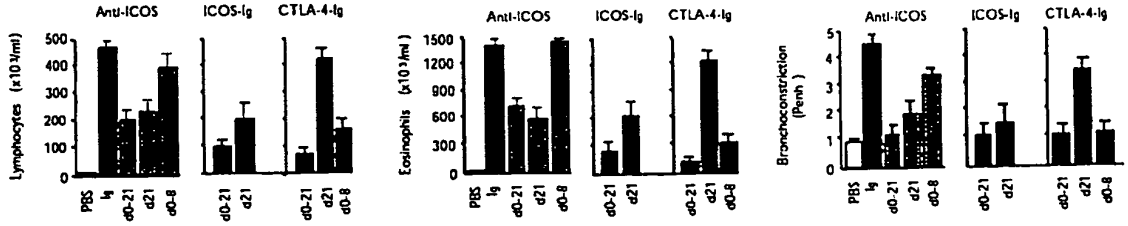

Figure 6. OVA-induced BAL leukocyte accumulation and AHR after ICOS neutralization at different time-points during allergic airway disease. The neutralizing mAb 12A8 (30 μg/mouse), CTLA-4–Ig (100 μg/mouse) or ICOS-Ig was administered *via* the i.p. route before OVA challenge on days 0, 8 and 15–21 or on days 0 and 8 or day 21 alone.

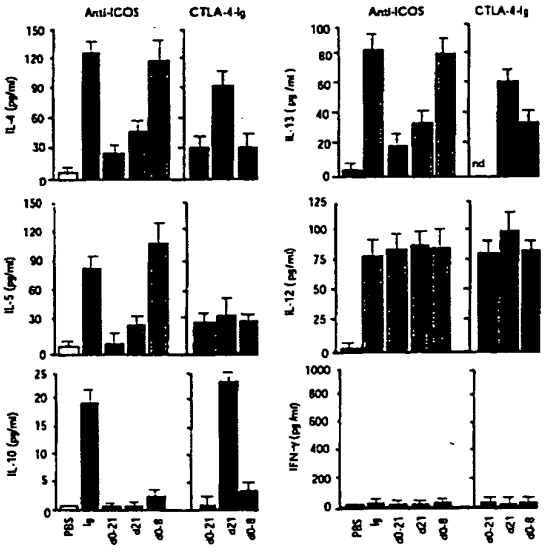

Figure 7. OVA-induced cytokine production in the BAL fluid after ICOS neutralization during allergic airway disease. Neutralizing mAb 12A8 (30 μg/mouse) and CTLA-4–Ig (100 μg/mouse) were administered *via* the i.p. route before OVA challenge on days 0, 8 and 15–21 or days 0 and 8 or day 21 alone. Data are mean±s.e.m. cytokine concentrations. Data from mice treated with PBS are shown in the open columns, data from control-treated animals are shown the filled columns. Data from ICOS-Ig– or CTLA-4–Ig-treated animals are shown in the shaded columns. nd, not determined.

up-regulate chemokine receptors in the draining lymph nodes. We prepared RNA from the lymph nodes of control Ig–treated or ICOS mAb–treated animals on day 21 before, and 1 h after, allergen challenge. Before allergen challenge on day 21, LNs contained germinal centers and elevated concentrations of antigen-specific IgE were present in the sera. Thus, the animals had mounted an effective response after immunization and aeroallergen challenge. By RT-PCR we determined that, after allergen challenge, CCR3, CCR4 and CCR8 mRNA was increased eight- to tenfold (**Fig. 8**). Treatment of animals with mAb 12A8 to ICOS prevented the up-regulation of these chemokine receptors. ICOS mAb treatment also inhibited up-regulation of IL-4 and IL-10 mRNA and increased expression of IFN-γ mRNA in the draining lymph nodes (**Fig. 8**).

Inhibition of costimulation and regulation of IgE

ICOS$^+$ cells can colocalize with B cells in the germinal centers of immunized mice, and ICOS plays a critical role in IL-4 production. Therefore we assessed next the impact of ICOS neutralization on the production of IgE. We found that, when administered on days 0–21, treatment with ICOS mAbs and CTLA-4–Ig abrogated IgE production. A similar reduction in IgE was observed when ICOS and CD28 were inhibited on days 0–8, although ICOS blockade was not as effective as CD28 blockade (**Fig. 9**).

Discussion

The interactions between CD28 and its ligands B7-1 and B7-2 are required for IL-2 production and T cell clonal expansion[23]. *In vivo* studies with animals deficient in CD28 or B7 genes or with the administration of the B7 antagonist CTLA-4–Ig have shown the essential role played by CD28 in regulating a number of immune responses[21–26]. However, although CD28-dependent mechanisms are implicated in primary T cell responses, secondary immune responses cannot be fully suppressed by administration of CTLA-4–Ig[27,28]. This suggests the existence of alternative or complimentary pathways for effective T cell activation. One possible candidate is ICOS. Through interactions with its ligand B7RP-1, ICOS provides a CD28-independent signal for IFN-γ and IL-4 production, but not IL-2 secretion, *in vitro*[9–11].

We have shown that *in vitro* ICOS blockade with a neutralizing mAb reduced expression of IL-4 and IL-10 and augmented secretion of IFN-γ. These data support published data obtained with ICOS-Ig *in vitro*, which show that ICOS can deliver a signal for immune deviation to T_H2 cytokine production that augments IFN-γ and reduces IL-4 production[29]. Although ICOS regulates T_H2 cytokine production, it is also preferentially expressed on T_H2 *versus* T_H1 cells, which supports the identification of the gene encoding murine ICOS as a T_H2-overexpressed gene[10]. However, although *in vitro* ICOS blockade facilitated immune deviation, production of IFN-γ *ex vivo* was not increased, despite reduced IL-4 and IL-10 secretion. This may be related to the use of complete Freund's adjuvant (CFA) as an adjuvant; because the contribution of ICOS to immune responses has been shown to be influenced by the adjuvant used[19].

Like ICOS, CD28 can promote T_H2 differentiation *in vitro*[13]. CTLA-4–Ig treatment *in vivo* can inhibit IL-4 production, but not IFN-γ secretion, during cutaneous leishmaniasis[30] and suppresses IL-4–dependent IgG1 production in a transgenic model of autoantibody production[25]. Similar conclusions were drawn using nonobese diabetic CTLA-4–Ig

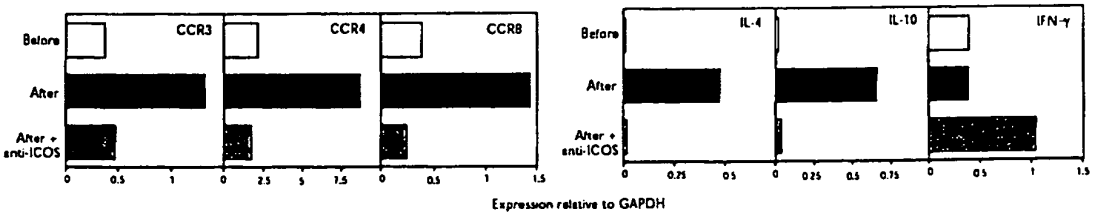

Figure 8. Regulation of chemokine receptor expression in the lymph nodes for ICOS. Lung-draining lymph nodes were removed on day 21, either before or 1 h after antigen challenge of control Ig–treated (open and filled bars, respectively) or anti-ICOS mAb 12A8–treated (shaded bars) animals. CCR3, CCR4 and CCR8 mRNA expression were measured by RT-PCR analysis. Expression of IL-4, IL-10 and IFN-γ mRNA were also evaluated. Each bar represents pooled data from 8–12 animals.

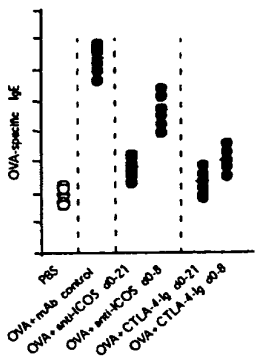

Figure 9. OVA-induced IgE production after ICOS neutralization and treatment with CTLA-4–Ig. Neutralizing mAb 12A8 (30 μg/mouse) or CTLA-4–Ig was administered *via* the i.p. route before each OVA challenge on days 0, 8 and 15–21 or on days 0 and 8, as indicated. Antigen-specific IgE was measured in the sera by specific ELISA. Data are the means±s.e.m. percentage difference of the OVA-specific absorbance values compared to PBS control animals. Each circle represents an individual mouse.

transgenic mice, which developed enhanced autoimmune diabetes that was associated with enhanced IFN-γ secretion, reduced IgG1 production and diminished T_H2 cytokine secretion[31]. These gave rise to the general concept that T_H2 responses are more dependent on costimulation than T_H1 responses. Thus, in some respects, CD28 and ICOS have overlapping functions that regulate the outcome of immune responses. However, CD28- and ICOS-mediated signaling differ in some important respects.

Although CD28 and ICOS both inhibit T cell expansion, CD28-mediated signaling is uniquely required for IL-2 production, whereas ICOS delivers a signal for secretion of tumor necrosis factor-α and IL-10 after superantigen administration[27]. In addition, during experimental allergic encephalomyelitis (EAE), ICOS-deficient mice exhibit markedly exacerbated responses[14], whereas CD28- or B7-deficient animals have attenuated disease[23]. The underlying mechanism(s) by which these molecules have opposing phenotypes in EAE is unclear, although one possible explanation is consistent with the ability of ICOS to facilitate T_H2 differentiation associated with reduced IFN-γ production. However, we have observed that, like the data obtained with ICOS-deficient mice, early therapeutic intervention increases clinical disease, which is associated with increased IFN-γ. Delayed treatment with mAbs to ICOS markedly impairs the onset of clinical symptoms during EAE, which is associated with reduced IFN-γ production[33]. Thus, the antigen experience of the T cell appears to be a critical factor in determining the outcome of ICOS blockade. Although ICOS engagement enhanced T_H2 and reduced T_H1 responses during differentiation, ICOS can regulate the production of both IL-4 and IFN-γ by recently activated T_H effector cells[10]. In addition, the nature of the immune response may also influence the role of ICOS. This is illustrated in another T_H1-mediated model where inhibition of ICOS, either with a blocking mAb or with the use of ICOS-deficient mice, prolongs rejection of cardiac allografts and is associated with reduced IFN-γ and IL-10 production[34].

To further address whether CD28 and ICOS have distinct or complimentary roles in regulating T_H2 responses, we next used a model of allergic lung inflammation driven by inhaled allergens[35,36]. The development of a lung eosinophilic inflammatory response is dependent on T cell–derived cytokines, including IL-4, IL-5 and IL-13[37]. CTLA-4–Ig, as well as ICOS blockade, inhibited T cell expansion and/or recruitment and reduced the recruitment of eosinophils to the lungs. These data support work by several investigators, who used either B7- or CD28-deficient animals or administered CTLA-4–Ig[24,38,39], and show that ICOS signaling is also critical in this process.

Although antigen-inexperienced $CD4^+$ T cells require B7-mediated signaling for IL-2 production, clonal expansion and the acquisition of effector function, optimal activation of recently activated T_H cells is B7-independent[40]. In addition, reactivation of memory cells occurs independently of both B7-1 and B7-2 [41]. Our data show marked differences in the requirement for ICOS *versus* CD28 engagement. Blockade of CD28 interfered with priming, but ICOS contributed to effector responses. Whether there are also distinct subpopulations of cells that secrete different patterns of cytokines that use either ICOS and/or CD28 remains to be determined.

The reduced cytokine concentrations obtained from the BAL fluid during the effector response may have been due to the failure of the cytokine-producing cells to migrate from the secondary lymph nodes to the lungs, a process regulated by chemokines and their receptors[37]. Our data on chemokine receptor expression raises the possibility that costimulatory signals regulate receptors that control the migration of effector T_HP cells to sites of allergic mucosal inflammation. The relationship between ICOS-mediated costimulation and the induction of chemokine receptors such as CXCR5 and CCR7, which distinguish between follicular T_H cells[42–44] and central or effector memory cells[45], remains to be determined.

ICOS-deficient mice are unable to generate germinal centers after protein immunization because of their impaired ability to up-regulate CD40 ligand[15]. $ICOS^+$ $CD4^+$ T cells were present within the B cell follicles of immunized and challenged, but not PBS-exposed, mice. This raises the possibility that $ICOS^+$ T cells receive signals through B7RP-1–expressing B cells and acquire the capacity to produce IL-4. In turn this would regulate the B cell class switch to IgE. In this context, ICOS blockade reduced IgE expression, although ICOS makes a smaller contribution to this process than CD28 does. Our data support published data obtained with ICOS-deficient mice that have a reduced ability to produce IgG1, IgE and also IgG2a after immunization with T-dependent B cell antigens[14–16] but produce normal antibody titers after immunization with T-independent antigens[16]. However, although ICOS plays a critical role in Ig production after immunization with soluble antigen alone or antigen in alum or incomplete Freund's adjuvant, the immune response to antigen in CFA is ICOS-independent[16]. Similarly, after infection with the nematode *Nippostrongylus braziliensis*, ICOS fails to regulate Ig secretion[46]. The precise basis for these differences is undetermined, but clearly the nature and/or strength of the immune stimuli greatly influence the dependence on ICOS-mediated costimulation.

We propose, therefore, the following model for the regulation and coordination of T cell–dependent inflammatory responses by CD28 and ICOS. T cells first become primed by a CD28-B7–dependent mechanism. Ligation of CD28 also up-regulates the chemokine receptor CXCR5[47], which allows these primed T_HP cells to migrate to the edge of the B cell rich follicle. Here they encounter antigen-bearing B cells that have also now accumulated in this area. These B cells then present peptides to primed antigen-specific T cells and, through B7RP-1, deliver a different signal that specifically facilitates T_H2 cytokine production. In turn, this up-regulates CD40 ligand, which delivers cognate signals to favor B cell expansion. B7RP-1–ICOS signaling could provide the proposed "step two of signal two"[48]. It would occur upon encounter with antigen-specific B cells that had endocytosed the same antigen for which the primed T_HP cells are antigen-restricted, thus ensuring the specificity of the immune response.

In addition to regulating this step of the immune response, B7RP-1–ICOS interactions would also deliver a signal for the up-regulation of CCR3, CCR4 and CCR8. As the ligands for these receptors—eotaxin,

MDC and I-309—are induced during allergic inflammation from epithelial cells and monocytes and have been implicated in mediating T_H2 accumulation in the lungs[35,36,49–51], ICOS-mediated costimulation of effector cells would then provide the signal for these effector cells to migrate from secondary lymph nodes into the airways. These data also provide evidence that suggests inhibition of signaling *via* the B7RP-1–ICOS pathway may represent a new target for the treatment of diseases such as allergic asthma, which are characterized by inappropriate T_H2 activation, without compromising host protective responses to pathogenic bacteria and viruses.

Methods

Monoclonal anti-ICOS generation. Wky rats were immunized *via* the intraperitoneal (i.p.) route with purified murine ICOS-Ig (100 μg) in CFA and boosted *via the* i.p. and subcutaneous routes. Splenocytes were fused with SP/2 myeloma cells and the resulting clones were screened for binding to ICOS-transfected Jurkat cells. Positive clones (12 of 600) were subcloned by limited dilution and isotyped with isotyping secondary antibodies (PharMingen, San Diego, CA). Identification of neutralizing mAbs were done by preincubating ICOS-transfected Jurkat cells (1×10^6/200 μl) with anti-ICOS (10 μg/ml) at room temperature for 30 min and then adding B7RP-1–Ig (2 μg/ml) to the mixture for another 30 min. ICOS–B7RP-1–Ig binding was detected by FACS with a phycoerythrin (PE)-conjugated goat anti-human Ig (PharMingen).

***In vitro* OVA stimulation.** Mice expressing the transgene encoding the DO11.10 αβ TCR, which recognizes chicken OVA amino acids 323–339 in association with I-A^{d} [52], were provided by D. Loh (Washington University, St Louis, MO). OVA-specific TCR-transgenic $CD4^+$ T cells were isolated from the spleen with negative-selection columns (R&D, Minneapolis, MN). Antigen-presenting cells (APCs) were prepared from mitomycin C–treated (50 μg/ml, 30 min) BALB/c splenocytes. OVA-specific $CD4^+$ T cells (2×10^5/ml) were stimulated with APCs (2×10^6/ml), the peptide OVA(323–339) (0.3 μg/ml), anti-ICOS (mAb 12A8 or 1C10, 10 μg/ml) or control rat Ig (10 μg/ml) for 5 days, washed and aliquoted into 96-well plates. Activated $CD4^+$ T cells (2×10^5/well) were restimulated with fresh APCs (2×10^5/well) and OVA (1 μg/ml) for another 2 days. The supernatants from these cultures were then collected and tested for cytokine production.

KLH antigen immunization. BALB/c mice that were 6–8 weeks old (Jackson Laboratory, Bar Harbor, ME) were immunized with KLH (1 mg/ml), emulsified in CFA (v/v 1:1), *via* the left footpad and base of the tail (50 μl/site). After 10 days, the draining lymph nodes were removed and cells were stimulated with KLH or, as a control, medium. The supernatants from these cultures were collected 48 h later and tested for cytokine production. Lymph nodes cells were removed after immunization with KLH for immunohistology on day 4 and day 7.

Induction of mucosal inflammation *in vivo*. C57BL/6J mice that were 6–8 weeks old (Jackson Laboratory) were housed in a specific pathogen–free mouse facility. The mouse model of lung inflammation used consisted of a sensitization phase (0.1 mg/mouse of i.p. OVA on day 0) (Sigma, St. Louis, MO) and an induction of the response phase (2% OVA for 5 min by aerosol on day 8 and 1% OVA for 20 min by aerosol on days 15–21). PBS (i.p. and/or aerosol) was administered to mice as a negative control. For the neutralizing experiments, mice also received 30 μg/mouse of anti-ICOS or 100 μg/mouse of ICOS-Ig or CTLA-4–Ig. Antibodies and fusion proteins were administered i.p. 30 min before OVA challenge on days 0, 8 and 15–21 or on days 0 and 8 or on day 21. OVA-treated control mice were injected with the same amount of control mAb at the same time-points as were administered during treatment. Three hours after OVA administration on day 21, mice were killed by CO_2 asphyxiation and lung leukocyte accumulation analyzed by bronchoalveolar lavages. AHR, expressed as enhanced pause (Penh) was measured 3 h after the last antigen challenge by recording respiratory pressure curves with whole-body plethysmography (Buxco Technologies, Sharon, CT) in response to inhaled methacholine (MCh, Aldrich, Milwaukee, WI) as described[36].

Measurement of gene expression by real-time PCR analysis. Total RNA from the lungs of OVA-treated mice or control littermates was extracted by single-step method with RNA STAT-60 (Tel-Test, Friendswood, TX). ICOS, CD28, CTLA-4, B7-1, B7-2, and B7RP-1 expression profiles were determined by RT-PCR (Taqman™, Perkin-Elmer, Norwalk, CT). Briefly, an oligonucleotide probe was designed to anneal to the gene of interest between two PCR primers. The probe was then fluorescently labeled with FAM (reporter dye) on the 5′ end and TAMRA (quencher dye) on the 3′ end. A similar probe and PCR primers were designed for GAPDH. The probe for this gene incorporated VIC as the reporter dye. PCR reactions were run that included the primers and probes for these two genes, as well as cDNA made from various cells and tissues. As the polymerase moved across the gene during the reaction, it cleaved the quencher dye from one end of each probe, which caused a fluorescent emission that was measured by the Sequence Detector 7700. The emissions recorded for each cDNA could then be converted into the amount of expression for the gene normalized to the expression of GAPDH. In addition, lung draining lymph nodes were removed on day 21 before and 1 h after antigen challenge, cDNA prepared as described and the amounts of CCR3, CCR4, CCR8, IL-4, IL-10 and IFN-γ determined. Animals were treated with anti-ICOS or Ig control 1 h before challenge.

Assessment of ICOS protein by immunohistochemistry. Protein expression was determined in noninflamed and inflamed mouse lung tissue samples using the mAb to ICOS and a modified biotin-avidin–staining method. ICOS, B cell and PNA^+ cell expression in the lymph nodes from KLH-immunized animals was also determined. Frozen sections were stained with anti-ICOS, then biotinylated anti–rat Ig and HRP–streptavidin. B cells were labeled with biotinylated B220 and detected with alkaline phosphate streptavidin and germinal centers detected with biotinylated PNA and avidin (both from Vector Laboratories, Burlingame, CA). All washes between antibody incubations were done in PBS Tween, and slides were mounted for microscopy in Fluoromount-G (Southern Biotechnology, Birmingham, AL).

Cytokine and IgE measurement. Serum and supernatant cytokine release was determined by ELISA. Sera from five different mice were analyzed in each group. Serial dilutions of samples were analyzed for IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ expression with commercial ELISA kits (Endogen, Boston, MA).

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Review

Murine models of asthma in understanding immune dysregulation in human asthma

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1. Introduction

Asthma is a chronic inflammatory disease that has been on the rise in recent years despite increased use of medication. Nevertheless, the fundamental mechanisms that underlie the development and perturbation of the asthmatic state remain elusive. Asthma is characterized by variable airflow obstruction, airway hyperresponsiveness (AHR) and airway inflammation. The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall with mast cells, lymphocytes and eosinophils. Although asthma is multifactorial in origin, recent advances suggest that asthma is an immune disease with a prominent role for T lymphocytes in the pathogenesis. In particular, $CD4^+$ T cells producing a Th2 pattern of cytokines (interleukin (IL)-4, IL-5, IL-13, IL-9) have been hypothesized to play a pivotal role in the pathogenesis of this disease (Gerblich et al., 1991; Robinson et al., 1992; Walker et al., 1992). These T cell-derived cytokines work in concert with chemokines and mediators released locally by the airway epithelium to orchestrate the recruitment and activation of the primary effector cells of the allergic response, the mast cell and the eosinophil. Activation of these effector cells results in the release of a plethora of inflammatory mediators that individually or in concert induce changes in airway wall geometry resulting in the symptoms of the disease.

Although considerable descriptive evidence suggests that $CD4^+$ T lymphocytes and Th2 cytokines are important in the pathogenesis of AHR in asthmatic humans, definitive proof is difficult to obtain in humans. Therefore, experimental animal models have been extremely useful in delineation of the role of $CD4^+$ T cells and T-cell-derived cytokines in the pathogenesis of asthma. The murine model has been a particularly valuable model because a wealth of immunologic reagents are available for the study of immune responses in this species. In addition, one of the attractive features of utilizing mice to study the pathogenesis of disease is the availability of over 200 well-characterized inbred strains and the ability

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to delete or overexpress specific genes through knockout and transgenic technologies.

2. Role of T cells in the pathogenesis of asthma

Direct evidence for a causal role for $CD4^+$ T cells in the development of AHR has been provided in mice devoid of CD4 T cells (Gonzalo et al., 1996) and in those treated with anti-CD4 mAb (Gavett et al., 1994). Definitive evidence for a pathogenic role for Th2 cells is provided by the fact that adoptive transfer of Th2 cells into the lungs of naive mice induces AHR and allergic inflammation (Li et al., 1996; Cohn et al., 1998). On the other hand, transfer of Th1 cells results in an inflammatory response, but no AHR (Cohn et al., 1998). Furthermore, studies in which administration of agents such as IL-12 and IFN-γ that inhibit Th2 cytokine production and stimulate Th1 pathways have been shown to prevent the development of antigen-induced AHR and inflammation in murine models (Lack et al., 1996; Gavett et al., 1995).

Despite the recognition that Th2 cytokines play a pivotal role in the development of the allergic diathesis, the exact mechanisms by which they induce asthma and AHR are still unknown. The major focus has been on the paradigmatic type 2 cytokines, IL-4 and IL-5. Both are thought to be central to the development of the allergic phenotype through their ability to drive IgE synthesis by B cells (Finkelman et al., 1988), and their critical involvement in the production, recruitment and activation of eosinophils (Wang et al., 1989). Indeed, early murine studies in which the levels of these cytokines were manipulated either through antibody blockade (Lukacs et al., 1994; Kung et al., 1995; Coyle et al., 1995) or gene targeting (Brusselle et al., 1995; Rankin et al.,1996; Foster et al., 1996) supported a role for these cytokines in allergen-driven pathophysiologic processes. However, more recent studies suggest that perhaps these two cytokines are not necessarily essential for the development of AHR (Coyle et al., 1995; Corry et al., 1998; Hogan et al., 1998). First, several groups showed that while antibody-mediated blockade of IL-4 during allergen sensitization ablates the development of allergic asthma, similar blockade of IL-4 prior to or during antigen challenge inhibits neither allergic inflammation nor AHR (Coyle et al., 1995; Corry et al., 1998). This strongly suggested that, while IL-4 plays its well-recognized immunoregulatory role in generating Th2 deviation in these models, it is not necessary for the expression of allergic asthma. This was further supported by the finding that transfer of Th2 cells derived from IL-4 deficient mice was still able to confer AHR (Cohn et al., 1998). Further evidence that neither IL-4 nor IL-5 were essential for the expression of AHR was provided by the finding that mice rendered deficient in both IL-4 and IL-5 still develop AHR in response to allergen sensitization and challenge (Hogan et al., 1998). Interestingly, Gavett et al. (1997) found that blockade of the IL-4 receptor alpha chain prior to antigen provocation in sensitized mice effectively inhibited AHR, eosinophilic accumulation, and mucus hyperplasia. Furthermore, Kuperman et al. (1998) showed that a deficiency in the signal transducer and activator of transcription-6 (STAT6) molecule, which mediates most of the cellular actions of both IL-4 and IL-13 (Zurawski et al., 1993), abolished antigen-induced eosinophilic inflammation and AHR. These results suggested that the steps distal to IL-4Rα ligation were clearly important in the development of allergic responses, but that IL-4 did not appear to be the ligand. As the T cell-derived cytokine, IL-13, also signals through this pathway, it was proposed that the effectiveness of IL-4 receptor blockade was due to inhibition of IL-13 mediated processes not those mediated by IL-4.

This hypothesis was supported by our recent finding that blockade of IL-13 at the time of antigen challenge in antigen-sensitized mice via administration of a soluble IL-13Rα2-Ig, which only binds IL-13, ablated antigen-induced AHR (Wills-Karp et al., 1998). Interestingly, the reversal of antigen-induced AHR was not associated with suppression in either IgE levels or BAL eosinophil numbers. However, antigen-induced mucus hyperplasia was inhibited by IL-13 blockade. Further proof of its importance in this response was provided by the finding that delivery of the recombinant cytokine to the lungs of naive animals reproduced many features of the allergic phenotype (i.e. AHR, eosinophilia, mucus hyperplasia) (Wills-Karp et al., 1998; Grunig et al., 1998). These findings have been corroborated by Zhu et al. (1999) using IL-13 transgenic mice. In addition, they demonstrated that chronic overexpres-

sion of IL-13 in the lungs of the transgenics also induced subepithelial fibrosis.

3. Role for IL-13 in human asthma

The relevance of these findings to human asthma is supported by the fact that IL-13 has been shown to be elevated in the lungs of asthmatics in several studies (Huang et al., 1995; Ying et al., 1997; Humbert et al., 1997; Till et al., 1997). Interestingly, elevations in IL-13 mRNA and protein levels appeared to be more associated with asthma than with atopy as levels were increased in the lungs of atopic and non-atopic asthmatic patients, but not in atopic-non-asthmatics when they were compared to normal subjects (Humbert et al., 1997). Of note, the *IL-13* and *IL-4* genes are located on human chromosome 5q31 in a region that has been linked with asthma (Marsh et al., 1994). Interestingly, a polymorphism in the IL-4R has recently been shown to be associated with asthma (Hershey et al., 1997).

4. Differential role of IL-4 and IL-13 in the allergic response

The reasons for the apparent differences in the contribution of IL-4 and IL-13 to the effector phase of the allergic response are not well understood. However, reasonable hypotheses include: (1) that there are differences in the kinetics of IL-4 and IL-13 production during the immune response to inhaled antigens; (2) that there are differences in the affinities of these receptors for IL-4 and IL-13; or (3) that these receptors induce different signaling pathways. The simplest of these explanations is that perhaps IL-4 is produced primarily during the differentiation phase of the response, and that IL-13 is either produced later or its production is sustained. Alternatively, the distribution and make-up of the IL-4/IL-13 receptor complex on lung cells may determine the binding affinity of the receptor complex for IL-4 or IL-13. There are currently two known IL-13-binding proteins referred to as IL-13Rα1 and IL-13Rα2 (Caput et al., 1996; Donaldson et al., 1998). The IL-13Rα2 chain specifically recognizes IL-13 with high affinity in the absence of a co-receptor. Its exact role in IL-13 signaling is unknown as it does not appear to serve a signaling function. The functional IL-13R complex is thought to contain the IL-13Rα1 and the IL-4Rα chains. This complex binds both IL-4 and IL-13. On the other hand, the unique IL-4 receptor is composed of the IL-4Rα and the common γ-chain (γc), a shared component of the receptors for IL-2, IL-7, IL-9, and IL-15. It has been shown that depending on the exact composition of these complexes, IL-4 and IL-13 will bind with different affinities and presumably compete for binding of the receptor complexes if both cytokines are present. Specifically, the presence of γc (which is primarily on hematopietic cells) lowers binding affinity of the complex for IL-13 and it is thought that the presence of the IL-13Rα2 may weaken binding of the complex for IL-4. Thus, differences in the make-up of the IL-4/IL-13 receptor complex on resident airway cells may bias the effector responses towards one or the other cytokine.

Signaling through IL-4/IL-13 complexes is thought to occur through the IL-4Rα chain as antibodies directed against the IL-4Rα chain inhibit the binding and the biologic activities of both IL-4 and IL-13. Both cytokines have been shown to activate the JAK-1 and Tyk-2 kinases and induce tyrosine phosphorylation of the IL-4Rα chain and the 170-kd insulin receptor substrate-2, which is the docking site for the Src homology domain containing the PI-kinase in lymphoid cells (Hilton et al., 1996). In contrast to IL-4, IL-13 does not induce the activation of JAK-3 kinase, which associates with the γc of the IL-4R complex after IL-4 binding. Phosphorylation of the IL-4Rα chain after binding of IL-13 or IL-4 results in the recruitment, phosphorylation, and nuclear translocation of STAT6 and the activation of *IL-13* and *IL-4* responsive genes (Zurawski et al., 1993). In the context of the allergic response, we have previously shown that STAT6 molecules are essential for the development of allergen-induced AHR (Kuperman et al., 1998). Thus, although some of IL-13's actions may be mediated via other signaling pathways, IL-13-induced AHR appears to be mediated via the STAT6 signaling pathway.

5. Potential mechanisms of IL-13-induced AHR

Although the exact mechanisms by which IL-13 induces AHR are currently not known, IL-13 has

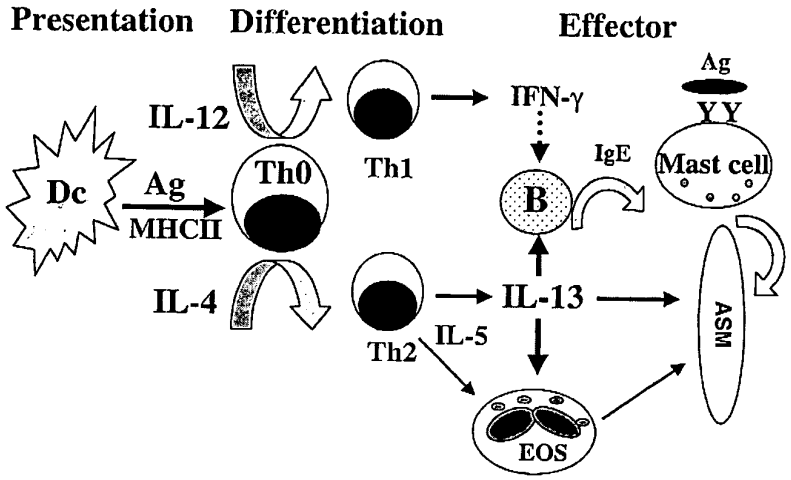

Fig. 1. Schematic representation of the potential mechanisms by which Th2 cells induce the allergic diathesis. In the lungs of asthmatic individuals stimulation of allergen-specific T cells by allergen-derived peptides presented by antigen-presenting cells (dendritic cells) in the context of class II MHC molecules results in differentiation of T cells into Th2 cytokine producing cells. Th2 cells produce IL-4, IL-13, and IL-5, which coordinately regulate the allergic response. IL-4 directs the differentiation of T cells towards a Th2 cytokine profile and acts as a growth factor for the expansion of these cells. IL-5 regulates the differentiation and egress of eosinophils from the bone marrow into the blood. IL-13, likely promotes the recruitment and activation of effector cells in the allergic response via binding to its receptor on numerous cells types such as B cells, eosinophils, and airway smooth muscle. Dc, dendritic cell; B, B cell; Ag, antigen; IgE, immunoglobulin E; MHCII, major histocompatibility complex II; ASM, airway smooth muscle; eos, eosinophil; IL-interleukin; Th, T helper cell.

several known functions, which are potentially important in the development of allergic airway disease: the induction of IgE production (Emson et al., 1998); activation of mast cells (Nilsson and Nilsson, 1995); the induction of VCAM-1 expression on vascular endothelium (Bochner et al., 1995); direct activation of eosinophils (Horie et al., 1997); and induction of chemokine production (Jordan et al., 1997) (Fig. 1). In addition, IL-13 may alter smooth muscle contractility either through one of the pathways highlighted above or independently. In this regard, Chen and Panettieri (1999) have recently reported that IL-13 can augment cholinergically induced contractions of tracheal smooth muscle in vitro suggesting that IL-13 may mediate AHR via direct effects on airway smooth muscle. Future studies will undoubtedly reveal the exact mechanism(s) by which IL-13 mediates the effector phase of the allergic response.

6. Conclusions

For over a relatively short period of time, a wealth of information regarding the immunological, physiological and molecular mechanisms of allergic responses has been derived from the use of murine models. Collectively, the studies to date suggest that Th2 cytokines are clearly important in the pathogenesis of allergic asthma. The individual Th2 cytokines likely have complementary roles in the induction and development of allergen-induced AHR. Specifically, IL-4 is necessary for the differentiation and expansion of Th2 cells, while IL-5 and IL-13 likely mediate the effector phase of the response by activating effector cells such as eosinophils, B cells and perhaps airway smooth muscle cells. The study of murine models should continue to provide insight into the immunological basis of allergic asthma and may ultimately lead to the development of immunotherapeutic strategies to reduce the morbidity and mortality associated with this disease.

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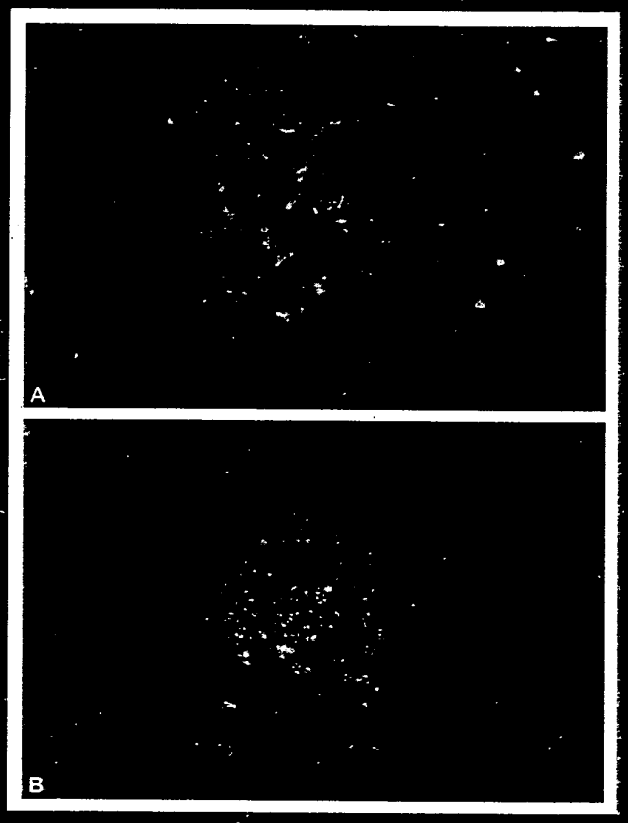

Development of Murine Allergic Asthma Is Dependent Upon B7-2 Costimulation[1]

Andrea M. Keane-Myers,* William C. Gause,‡ Fred D. Finkelman,† X.-d. Xhou,‡ and Marsha Wills-Karp[2]*

Allergic asthma is thought to be mediated by CD4$^+$ T lymphocytes producing the Th2-associated cytokines, IL-4, and IL-5. Recently, the costimulatory molecules B7-1 and B7-2, which are expressed on the surface of APC, have been suggested to influence the development of Th1 vs Th2 immune responses. We examined the in vivo role of these costimulatory molecules in the pathogenesis of Th2-mediated allergen-induced airway hyperresponsiveness in a murine model of asthma. In this model, OVA-sensitized A/J mice develop significant increases in airway responsiveness, pulmonary eosinophilia, and pulmonary Th2 cytokine expression following aspiration challenge with OVA as compared with PBS-control animals. Strikingly, administration of anti-B7-2 mAb to OVA-treated mice abolished allergen-induced airway hyperresponsiveness, pulmonary eosinophilia, and elevations in serum IgG1 and IgE levels. Anti-B7-2 treatment of OVA-treated mice reduced both total lung IL-4 and IL-5 mRNA and bronchoalveolar lavage fluid IL-4 and IL-5 protein levels, with no significant changes in IFN-γ message or protein levels. In contrast, treatment with anti-B7-1 mAbs had no effect on allergen-induced airway hyperresponsiveness, IgE production, or cytokine production, however, it significantly suppressed pulmonary eosinophilia. We conclude that B7-2 provides the necessary costimulatory signal required for the development of in vivo allergic responses to inhaled allergen exposure. *The Journal of Immunology,* 1998, 160: 1036–1043.

Allergic asthma is a disease characterized by airway hyperresponsiveness, pulmonary inflammation, and elevated serum IgE levels. Increasing evidence suggests that T lymphocytes, in particular CD4$^+$ T cells of the Th2 phenotype, play a pivotal role in the development of the airway hyperresponsiveness and the eosinophilic inflammatory response common in asthma (1–3). Elevated IL-4 and IL-5 levels in bronchial biopsies (3), bronchoalveolar lavage (BAL)[3] cells (1, 2), and blood (2) of allergic asthmatic patients have been noted. Since these cytokines promote eosinophil chemotaxis (4), activation (5), and survival (6), as well as IgE production by B cells (7), this cytokine pattern has been thought to be important in human allergic asthma.

We have recently provided additional support for this hypothesis in a murine model of Ag-induced airway hyperresponsiveness and pulmonary eosinophilia in which allergic responses are CD4$^+$ T cell dependent (8) and associated with increases in Th2 cytokines in the lung (9). In addition, we demonstrated that administration of rIL-12, a cytokine important in Th1 cell differentiation, both prevented and reversed the development of allergic airway responses in mice (9). Despite considerable evidence suggesting that T lymphocytes play a pivotal role in the pathogenesis of asthma, the molecular signals that direct the differentiation of naive T cells into pathogenic Th2 cytokine-producing cells in the lung in response to inhaled allergens are not well understood.

CD4$^+$ T cell activation requires two distinct signals from APC (10). The first signal, which confers specificity, is provided by the interaction of the TCR with MHC II complexes on APC. A second costimulatory signal can be provided by APC-borne ligands for the CD28 and CTLA-4 receptors on T cells. TCR ligation in the absence of costimulation induces Ag-specific T cell anergy (11). The ligands for CD28 and CTLA-4 are B7-1 (CD80) and B7-2 (CD86) (12, 13). Blockade of the B7/CD28 pathway with CTLA4Ig, a soluble fusion protein (14), has been shown to effectively inhibit T cell activation in vitro (15, 16) and in vivo (17–22). Recently, some studies have suggested that B7/CD28-CTLA-4 interactions may not only be important in T cell activation and IL-2 production, but may also play a role in T cell differentiation with B7-1 favoring development of Th1 cells and B7-2 favoring Th2 cells (22–24). In contrast, other studies have suggested that B7-1 and B7-2 molecules can substitute for each other during Th2 differentiation (25, 26). Few studies have examined the potential role of these costimulatory molecules in the development of T cell-dependent allergic airway responses.

In the present study, we examined the relative contribution of B7-1 and B7-2 to the development of Th2-mediated allergic airway responses in an in vivo murine model of asthma, which we have previously described (8, 9). Our results demonstrate that B7-2 costimulatory molecules are required for the development of a type 2 cytokine pattern as well as the development of allergic airway responses. In contrast, treatment with anti-B7-1 did not block the course of the type 2 immune response.

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[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage; HPRT, hypoxanthine guanine phosphoribosyltransferase.

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Materials and Methods

Animals

Six-week-old male A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were housed in a laminar flow hood in a virus-free animal facility for the duration of the experiments. The studies reported here conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education and Welfare (National Institutes of Health) guidelines for the experimental use of animals (n = 6–8 mice/experimental group).

Special reagents

Abs used for the in vivo cytokine intervention experiments included rat anti-mouse B7-2 (GL1) (27), hamster anti-mouse B7-1 (16-10A1) (28), control isotype-matched rat IgG2a (GL117), and control normal hamster IgG, which were prepared as previously described (29).

Effects of B7 blockade on allergic airway responses to Ag challenge

Mice were sensitized by i.p. injection of OVA (10 μg in 0.3 ml PBS) or PBS alone (controls). Two weeks later, mice were anesthetized with a mixture of ketamine and xylazine (45 and 8 mg/kg, respectively) and challenged by aspiration. Specifically, mice are placed on a board in a supine position. The animal's tongue extended with lined forceps and 50 μl of a 1.5% solution of OVA or an equivalent volume of PBS (control) is placed on the back of the tongue. We have examined the deposition pattern of the Ag delivered in this manner by using Evans blue dye. We find that a small amount of the Ag is deposited in the trachea and the remainder is deposited in the airways. No Ag is detected in the esophagus or stomach.

OVA-treated (both sensitized and challenged with OVA) and PBS-treated mice (both sensitized and challenged with PBS vehicle) were injected i.v. with anti-B7-1 (100 μg), anti-B7-2 (100 μg), or a combination of both anti-B7-1 and anti-B7-2 mAbs (total of 200 μg) 24 h prior to OVA or PBS challenge. Control animals were administered either 100 μg hamster IgG, 100 μg IgG2a (GL117), or 100 μg of both hamster IgG and IgG2a (GL117). On the day of the challenge, Abs (100 μg/mouse) were administered with the PBS or OVA by aspiration in a total volume of 50 μl. Ninety-six hours after challenge, airway responsiveness to i.v. acetylcholine challenge was determined, the number of inflammatory cells in BAL fluids was determined, lungs were saved for measurement of cytokine mRNA levels, and blood was taken for analysis of Ig levels.

Airway responsiveness measurements

Airway responsiveness to i.v. acetylcholine challenge was measured as previously described with minor modifications (8, 9). Briefly, mice were anesthetized with sodium pentobarbital (17.5 mg/ml), intubated with a 20-gauge tracheal cannula, and ventilated at a rate of 120 breaths/min with a constant tidal volume of air (0.2 ml). Airway pressure was measured with a pressure transducer via a port of the tracheal cannula. Muscle paralysis was provided by i.v. administration of decamethonium bromide (25 mg/kg). After establishment of a stable airway-pressure recording, acetylcholine was injected i.v. (50 μg/kg) and the changes in airway pressure were recorded. Airway responsiveness was defined by the time-integrated change in peak airway pressure (airway pressure-time index; cm H_2O per s).

Assessment of airway inflammation

After airway responsiveness measurements, lungs were lavaged thoroughly with 1 ml of HBSS solution without calcium or magnesium plus 10% FBS. The lavage fluid was centrifuged (300 × g × 10 min), the supernatant was removed for cytokine analysis, and the cell pellet was resuspended in 1 ml of HBSS solution plus 10% FBS, and counted with a hemocytometer. Slide preparations were stained with Diff-Quick (Baxter, McGaw Park, IL) and BAL cell differential percentages were determined based on light microscope evaluation of >500 cells/slide.

Quantitation of cytokine mRNA levels in the lung

RNase-free plastic and water were used throughout the assay. Tissues were homogenized in RNAzol B (Cinna/Biotecs, Friendswood, TX) at 50 mg of tissue/ml. Purified RNA (10 μg) was subjected to electrophoresis on a 2% formaldehyde gel containing ethidium bromide (30). The gel was photographed and individual lanes were examined for the presence of 18S and 28S ribosomal bands, the absence of RNA degradation, and the quantity of RNA loaded onto each lane.

The procedures for reverse transcription and PCR were previously reported (30) and are briefly described here. RNA samples were reverse transcribed with Superscript RT (Bethesda Research Laboratories, Rockville, MD), and cytokine-specific primers were used to amplify selected cytokines (30). For each cytokine, the optimum number of cycles (i.e., the number of cycles that would produce a detectable quantity of cytokine product DNA that was directly proportional to the quantity of input mRNA) was determined experimentally. To verify that equal amounts of undegraded RNA were added in each RT-PCR reaction within an experiment, the "housekeeping gene," hypoxanthine guanine phosphoribosyltransferase (HPRT), was used as an endogenous internal standard, and amplified with specific primers at the number of cycles at which a linear relationship between input RNA and final HPRT product was detected. Although HPRT values did not usually vary more than two- to threefold, values for specific cytokines were normalized to HPRT values. Amplified PCR product was detected by Southern blot analysis and the resultant signal was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Mean values are expressed relative to the means of the PBS-challenged animals, which were arbitrarily given a value of 1.

Quantitation of cytokine protein levels in BAL fluid

After airway measurements, mice were lavaged thoroughly with 1 ml of HBSS solution without calcium and magnesium plus 10% FBS. The lavage fluid was centrifuged, and aliquots of the supernatants were stored without further treatment at −80°C until analyzed by ELISA. ELISAs for IL-4, IL-5, and IFN-γ were conducted using matching Ab pairs obtained from PharMingen (San Diego, CA), according to the manufacturer's instructions. The following Ab pairs were used for ELISA detection of IL-4, IL-5, and IFN-γ, respectively: BVD4-1D11 and BVD6-24G2; TRFK5 and JES1-39D10; R46A2 and XMG1.2. OD readings of samples were converted to picograms per milliliter using values obtained from standard curves generated with varying concentrations of recombinant IL-4, IL-5 and IFN-γ (2000 pg/ml-5 pg/ml). The limit of detection was 5 pg/ml for each assay.

OVA-specific IgG1 and IgG2a ELISA assays

Sera were obtained from blood taken during exsanguination of the animals after airway measurements. IgG subclass specific ELISAs were used to quantitate OVA-specific IgG1 and IgG2a Ab levels in serum. Briefly, 96-well Corning ELISA plates were coated with 50 μl of OVA (100 μg/ml) in HBSS overnight at room temperature. Sixteen hours later, wells were blocked with the addition of PBS-10% FBS (200 μl/well) for 2 h at room temperature. Following blocking, the plates were washed with PBS-Tween-20, sera were added (100 μl/well of a 1:100 dilution in PBS-1% FBS), and plates were incubated overnight at 4°C. Plates were then washed with PBS-Tween and incubated with biotin-conjugated anti-mouse IgG1 (1:2000; γ_1 chain specific) or anti-mouse IgG2a (1:2000; γ_{2a} chain specific; PharMingen) for 1 h at room temperature. After washing, plates were blotted dry and developed with 100 μl of an avidin peroxidase solution (ABTS, Kirkegaard and Perry, Gaithersburg, MD). Plates were read at 405 nm.

Quantitation of total serum IgE

Sera were obtained from blood taken during exsanguination of the animals after airway measurements and 100 μl (1:50 dilution in 1% FBS in PBS) was added/well. An IgE-specific ELISA was used to quantitate total IgE Ab levels in serum using matching Ab pairs (R35-72 and R35-92) obtained from PharMingen according to the manufacturer's instructions. OD 405 readings of the samples were converted to picograms per milliliter using values obtained from standard curves generated with varying concentrations of IgE and the final concentration was obtained by multiplying by the dilution factor.

Data analysis

Data are summarized as mean ± SE. The statistical analyses of the results were performed by analysis of variance using Fisher's least significant difference test for multiple comparisons. Probability values <0.05 were considered significant.

Results

B7-2 blockade ablates airway hyperresponsiveness and pulmonary inflammation

As B7-1 and B7-2 molecules are thought to be important in Th cell differentiation, we sought to determine the relative contribution of B7-2 and B7-1 to the development of Th2-mediated Ag-induced airway hyperresponsiveness and inflammation. As previously reported (8, 9), airway reactivity to i.v. acetylcholine challenge in OVA-treated mice was significantly increased

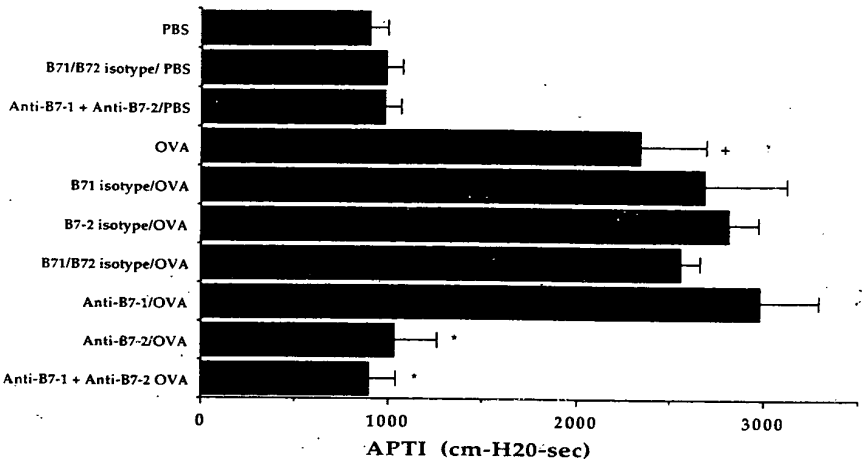


FIGURE 1. B7-2 blockade inhibits Ag-induced airway hyperresponsiveness to i.v. acetylcholine in mice 96 h after a single challenge with OVA or PBS. OVA-sensitized mice were injected i.v. with either anti-B7-1 (100 μg), anti-B7-2 (100 μg), or a combination of both Abs (200 μg) 24 h prior to aspiration challenge with OVA, respectively. Control animals were administered either 100 μg hamster IgG, 100 μg IgG2a (GL117) or 100 μg of both hamster IgG and IgG2a (GL117). On the day of the challenge, either specific Abs or isotype-matched control Abs (100 μg/mouse) were administered with the PBS or OVA by aspiration in a total volume of 50 μl. Values shown are means + SE of six to eight animals per group. $^{+}p < 0.05$ compared with PBS group; $^{*}p < 0.05$ compared with OVA + respective isotype control mAb-treated groups.

compared with that in PBS-treated mice (Fig. 1). Strikingly, anti-B7-2 treatment completely ablated airway hyperresponsiveness to acetylcholine in OVA-treated mice as compared with airway responses of OVA-treated mice receiving the isotype-matched Ab (GL117). In marked contrast, anti-B7-1 mAb treatment had no significant effect on airway reactivity in OVA-treated mice as compared with their controls (OVA + hamster IgG). Isotype-matched control Ab (i.e., hamster IgG or rat IgG2a (GL117)) administration had no significant effect on airway reactivity in OVA-treated mice. Combining anti-B7-1 and anti-B7-2 mAb treatments suppressed airway responses to the same degree as anti-B7-2 mAb treatment alone. These results demonstrate the importance of B7-2 molecules in the functional airway responses to T cell activation by inhaled Ags.

Examination of the cellular composition of BAL fluids revealed that the majority of cells in the BAL fluid of PBS control animals were alveolar macrophages (Fig. 2*A*). Following OVA sensitization and challenge no significant increases in BAL macrophages were observed, however, there were marked increases in the number of BAL eosinophils (Fig. 2*B*). Anti-B7-2 treatment of OVA-treated mice resulted in virtual ablation of eosinophils in the BAL, while anti-B7-1 partially suppressed the number of eosinophils recovered in the lavage fluid. The combination of anti-B7-1 and B7-2 mAbs did not result in any additional significant inhibitory effect on eosinophil numbers over that observed with anti-B7-2 treatment alone. All three isotype-matched control Abs had a tendency to reduce alveolar macrophage numbers although not significantly; however, they had no significant effect on eosinophil numbers in the BAL.

B7-2 costimulation is required for Th2 cell differentiation

To determine the role of B7 molecules in T cell differentiation and cytokine production, we examined the effects of blockade of B7-1, B7-2, or both on IL-4, IL-5, and IFN-γ mRNA and protein levels. Detectable mRNA and protein levels of IL-4, IL-5, and IFN-γ were observed in PBS-treated mice (Figs. 3, 4, and 5). As we have previously demonstrated (9), following Ag challenge significant increases in both mRNA and protein levels of the type 2 cytokines, IL-4 and IL-5, were observed, whereas no significant increases in the type 1 cytokine, IFN-γ were found. Ag challenge also induced significant increases in IL-10 mRNA levels as compared with PBS controls (Fig. 6). Anti-B7-2 or the combination of anti-B7-1 and anti-B7-2 mAbs significantly suppressed Ag-induced increases in protein levels of IL-4 and IL-5 as compared with their respective isotype Ab controls, while having no effect on IFN-γ levels. In marked contrast, anti-B7-1 did not significantly alter IL-4, IL-5, or IFN-γ mRNA or protein levels in OVA-treated mice as compared with hamster IgG treatment. Interestingly, the combination of anti-B7-1 and anti-B7-2 Abs significantly increased BAL IFN-γ protein levels in PBS-treated mice. Isotype control Ab administration to OVA-treated mice had no significant effect on BAL IL-4 and IL-5 protein levels as compared with OVA-treated mice receiving no Ab treatments. These results demonstrate that B7-2 provides costimulation for the differentiation of T cells into type 2-producing cells in response to inhaled Ag, and suggests that B7-1 does not contribute to the immune response in this model, at least to the extent that the dose of anti-B7-1 Ab used inhibited B7-1 interactions.

Effects of B7 blockade on serum Ig levels

To examine the role of B7 molecules in humoral responses to inhaled OVA exposure, we measured OVA-specific levels of IgG1 and IgG2a and total IgE in serum samples from animals receiving Abs to B7-1, B7-2, or both. Administration of anti-B7-2 mAb to Ag-treated mice resulted in significant suppression of OVA-specific IgG1 when compared with mice treated with the isotype control Ab GL117, while having no effect on IgG2a levels (Fig. 7). On the other hand, anti-B7-1 did not affect either IgG1 or IgG2a levels. Combined mAb treatment did not result in any significant inhibitory effect on Ig levels over that observed with anti-B7-2 treatment alone. Isotype-matched control mAb treatment had no effect on OVA-specific serum levels of IgG1 or IgG2a.

OVA sensitization and challenge resulted in significant elevations in total serum IgE levels as compared with PBS controls (Fig. 8). Anti-B7-2 mAb treatment abolished OVA-induced increases in IgE levels when compared with isotype control Ab-treated mice, while anti-B7-1 had no effect. Isotype-matched control Abs had no significant effect on IgE levels in either PBS- or OVA-treated mice. As IgE Ab production is IL-4 dependent, these results lend further support for the role of B7-2 costimulatory molecules in

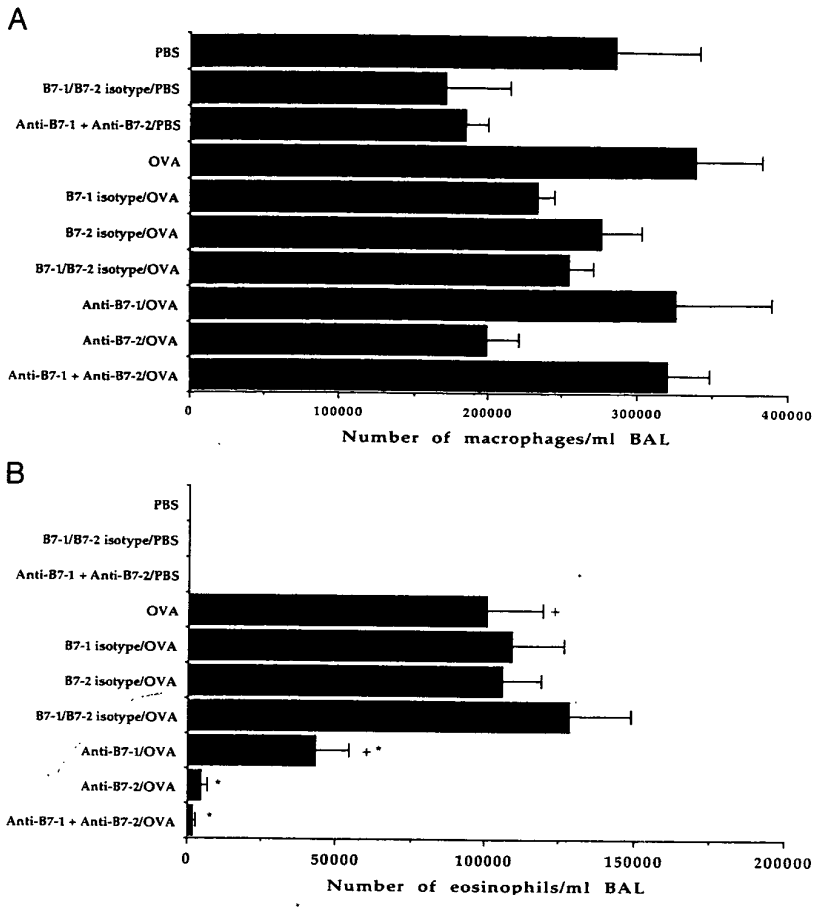


FIGURE 2. Effects of B7 blockade on numbers of BAL macrophages (*A*) and eosinophils (*B*) recovered from mice 96 h after a single OVA or PBS challenge. Values shown are mean + SE of 6 to 8 mice/group. Mice were treated as described in Figure 1. $^{+}p < 0.05$ compared with PBS group; $^{*}p < 0.05$ compared with OVA + respective isotype control mAb-treated groups.

type 2 cytokine production as well as in the development of allergic responses.

Discussion

Allergic asthma is characterized by airway hyperresponsiveness, eosinophilic inflammation and elevated serum IgE levels. Several studies have shown that this disease is associated with elevated T lymphocytes in the lungs and a Th2 pattern of cytokine production (1–3). Utilizing a murine model, we have previously demonstrated that the development of allergic responses to allergens is $CD4^{+}$ T cell dependent (8) and ameliorated by the addition of agents that either directly block Th2 cytokine activity or that prevent the expansion of type 2 cytokine-producing cells (9, 31). Studies in several experimental systems have demonstrated the importance of IL-4 and IL-5 in the development of airway hyperresponsiveness, eosinophilia, and elevations in IgE (32, 33).

It has recently been demonstrated that T cell activation requires multiple signals in addition to those conveyed through interaction of the TCR with MHCII complexes on APC (10). One of the most well characterized costimulatory signaling pathways involves the CD28 and CTLA-4 molecules that are expressed on T cells and their ligands, B7 molecules that are expressed on APC (11–13). We have recently demonstrated that CTLA4Ig, a fusion protein that inhibits the interaction of B7 molecules with both CD28 and CTLA-4, ablated the development of airway hyperresponsiveness, eosinophilic inflammation, and elevations in serum IgG1 and IgE Abs when administered either prior to sensitization or prior to local lung challenge (34). Inhibition of these responses was associated with suppression of Th2 cytokines without any changes in the Th1 cytokine, IFN-γ. These studies demonstrated that B7 molecule interaction with either CD28 or CTLA-4 was important both in the initial sensitization step and in the subsequent challenge response in the lung. Our findings support that of previous studies using similar murine models (35, 36). To date at least two members of the B7 family have been identified, namely B7-1 (CD80) and B7-2 (CD86) (12, 13). Considerable controversy exists as to whether these molecules are interchangeable or mediate distinct functions through CD28 (22–26). Some studies have supported the concept that they mediate distinct functions and moreover that B7-1 is important in Th1 cell differentiation and that B7-2 molecules are important in Th2 cell differentiation (22–24). However, previous in vitro studies have suggested that either B7-2 costimulation promotes the differentiation of naive T cells toward a Th2 phenotype (24) or that B7-1 or B7-2 interactions with CD28 can support Th2 cell differentiation (25). The present study was undertaken to determine whether the ligation of B7-1, B7-2, or both molecules was required for development of a type 2 mucosal immune response to Ag exposure in a murine model.

Our results demonstrate that B7-2, but not B7-1, costimulation is necessary for the development of allergic responses to inhaled Ags in this murine model. Strikingly, B7-2 blockade completely eliminated Ag-induced airway hyperresponsiveness, eosinophilic inflammation, and elevations in serum IgE levels. In conjunction with its inhibitory effects on these functional responses, anti-B7-2 mAb ablated Ag-induced increases in Th2 cytokines in BAL

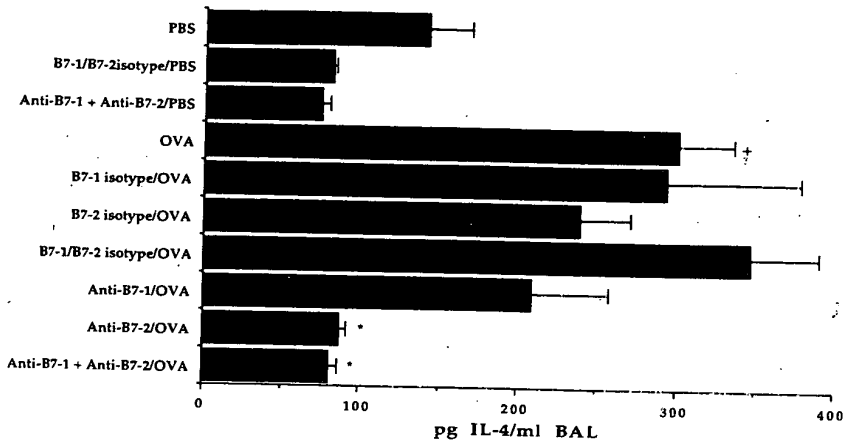


FIGURE 3. Effect of B7 blockade on IL-4 protein levels in BAL supernatants of mice 96 h after a single OVA or PBS aspiration challenge. PBS- or OVA-challenged mice were treated with anti-B7-1, anti-B7-2, or a combination of both Abs as described in Figure 1. Protein levels were analyzed using ELISAs as described in *Materials and Methods*. After subtracting background absorbance, OD readings were converted to picograms per millimeter by comparison with standard curves. Results shown are means + SE of cytokine protein levels for six to eight mice in each group. $^{+}p < 0.05$ compared with PBS group; $^{*}p < 0.05$ compared with OVA + respective isotype control mAb-treated group.

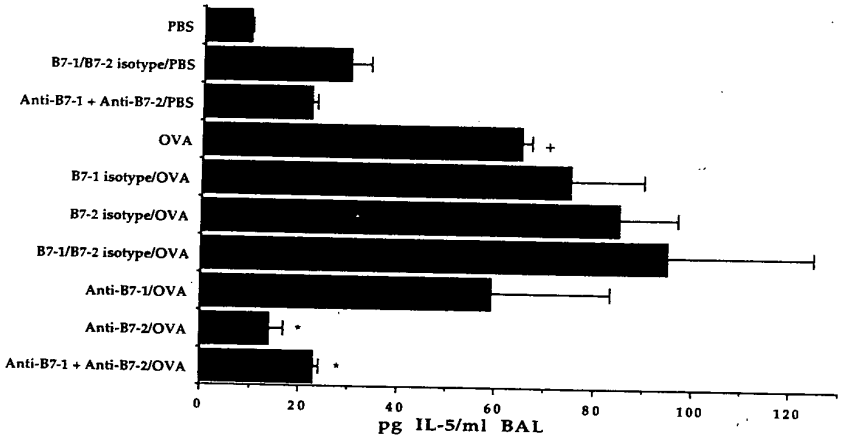
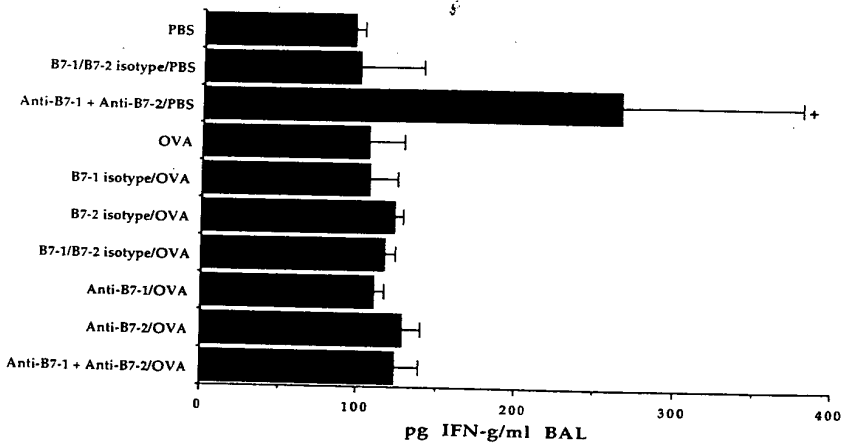


FIGURE 4. Effect of B7 blockade on IL-5 protein levels in BAL supernatants of mice 96 h after a single OVA or PBS aspiration challenge. PBS- or OVA-challenged mice were treated with anti-B7-1, anti-B7-2, or a combination of both Abs as described in Figure 1. Protein levels were analyzed using ELISAs as described in *Materials and Methods* and in the legend to Figure 3. Results shown are means + SE of cytokine protein levels for six to eight mice in each group. $^{+}p < 0.05$ compared with PBS group; $^{*}p < 0.05$ compared with OVA + respective isotype control mAb-treated group.

FIGURE 5. Effect of B7 blockade on IFN-γ protein levels in BAL supernatants of mice 96 h after a single OVA or PBS aspiration challenge. PBS- or OVA-challenged mice were treated with anti-B7-1, anti-B7-2, or a combination of both Abs as described in Figure 1. Protein levels were analyzed using ELISAs as described in *Materials and Methods* and in the legend to Figure 3. Results shown are means + SE of cytokine protein levels for six to eight mice in each group. $^{+}p < 0.05$ compared with PBS group; $^{*}p < 0.05$ compared with OVA + respective isotype control mAb-treated group.



fluids, with no effect on IFN-γ levels. As there were no significant increases in IFN-γ after Ag challenge, it is not surprising that anti-B7-2 mAb treatment did not affect IFN-γ levels. In contrast, anti-B7-1 blockade did not result in suppression of Ag-induced airway hyperresponsiveness, IgE production, or Th2 cytokine production. These results provide evidence that B7-2 costimulation can play an essential role in the development of allergic asthma.

Although B7-2 was required for the development of this type 2 allergic immune response, blocking B7-2 interactions did not cause immune deviation toward increased IFN-γ expression, nor did blocking B7-1 interactions promote a more severe allergic response. Similarly, we have shown that CTLA4Ig treatment of OVA-sensitized and challenged A/J mice suppressed Ag-induced Th2 cytokine production, but did not alter IFN-γ levels (37). Our results are in contrast to those of Tsuyuki et al.

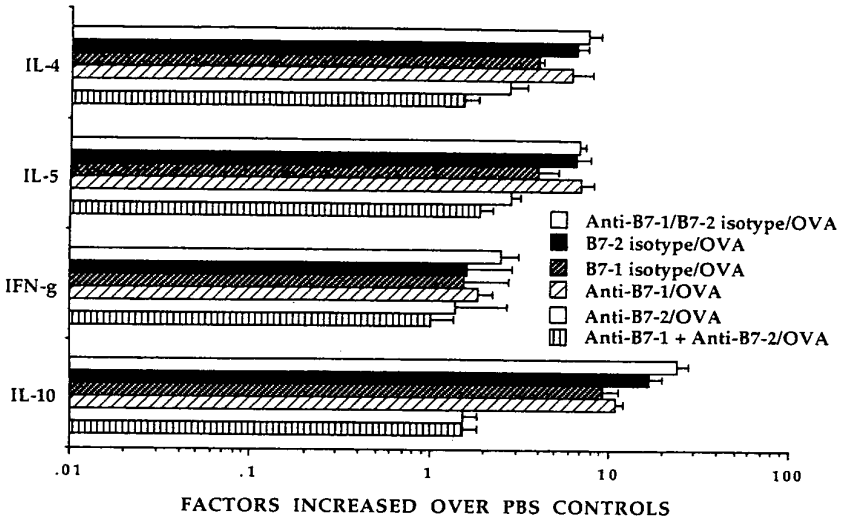


FIGURE 6. Effects of B7-1 and B7-2 blockade on IL-4, IL-5, IFN-γ, and IL-10 mRNA expression in the lungs of mice 96 h after OVA challenge. Mice were treated as described in Figure 1. RNA was extracted from lungs and purified using the RNAzol B method, reverse transcribed into cDNA, and then subjected to varying cycles of PCR as described in *Materials and Methods*. Amplification of HPRT was performed as a control for the total amount of cDNA used in PCR. Data are expressed as the mean + SE of the fold increase over control PBS values ($n = 4$).

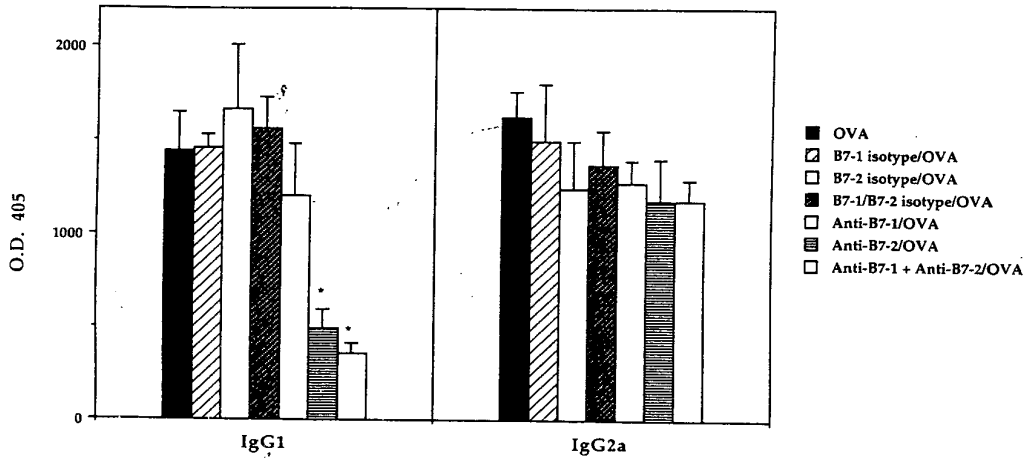


FIGURE 7. Effects of B7 molecule blockade on OVA-specific serum levels of IgG1 and IgG2a. Mice were treated as shown in Figure 1. OD values from PBS-challenged control mice were at background levels (50 ± 0.05). Values shown are the mean + SE of Ab levels from six to eight mice per group. $^*p < 0.05$ as compared with respective isotype control mAb-treated OVA group.

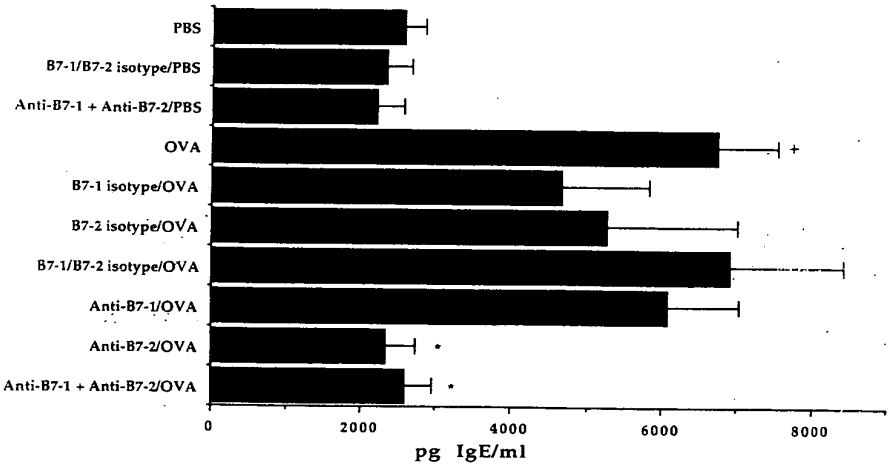


FIGURE 8. B7-2 blockade inhibits Ag-induced increases in total serum IgE levels. Mice were treated as described in Figure 1. Values are mean + SE of IgE levels from six to eight mice per group. $^+p < 0.05$ compared to PBS group; $^*p < 0.05$ compared with respective isotype control mAb-treated OVA group.

(35) who demonstrated using a similar Ag exposure model with SV129 mice that anti-B7-2 mAb administration did in fact induce increases in IFN-γ levels in in vitro-activated lung T cells. These differences may reflect the inherent susceptibility of the A/J strain to the development of Th2 responses and that IFN-γ production or pathways associated with IFN-γ production are

deficient in these mice. Consistent wit[redacted] is our finding that if these animals are given exogenous IL-12 they will produce IFN-γ, which effectively ablates the development of the allergic phenotype (9). Another potential difference is that in our study cytokines were measured in BAL fluids taken at the time of measurement of allergic airway responses whereas in their study, IFN-γ levels were derived from isolated lung cells stimulated in vitro with anti-CD3 Abs.

Our studies demonstrate that B7-2, but not B7-1, costimulation is necessary for T cell help leading to B cell activation and secretion of IgG1 and IgE. Since these Abs are associated with Th2 cell effector function, they provide further evidence that B7-2 costimulation is required for IL-4 production and the development of a type 2 immune response (7). These observations are consistent with our previous finding that CTLA4Ig prevents the development of humoral responses in allergen-challenged A/J mice (34). Consistent with the lack of increase in IFN-γ production in anti-B7-2-treated animals, IgG2a levels, which are thought to be IFN-γ dependent, were not affected by anti-B7-2 treatment. The lack of involvement of B7-1 in OVA-induced Ab production was also demonstrated using Y100F-Ig, which recognizes B7-1 not B7-2, in a murine model of Ag-induced airway eosinophilia (37).

One interesting finding was that CTLA4Ig administration to mice before Ag sensitization resulted in blockade of OVA-specific Abs of all isotypes studied (IgE, IgG1, and IgG2a), whereas when it was administered prior to Ag challenge it suppressed only IgE and IgG1 with no effect on IgG2a levels. Administration of anti-B7-2 Abs reproduced the effect of CTLA4Ig on Ab production when it was given prior to local lung challenge. These results suggest that perhaps IgE and IgG1 Ab increases were due to Ag presentation in the lung following local Ag challenge and that a more mixed response occurs during systemic sensitization. This is consistent with our observation that IgG1 and IgE Ag production is markedly elevated following local Ag challenge as compared with that following systemic sensitization alone (M. Wills-Karp and A. M. Keane-Myers, unpublished observations). This hypothesis is supported by a recent report by Chvatchko et al. (38) in which they demonstrated the formation of germinal centers within the lung parenchyma, which predominantly produced OVA-specific IgG1 and IgE in mice challenged intratracheally with OVA.

Surprisingly, anti-B7-1 treatment of OVA-sensitized and challenged animals partially suppressed OVA-induced increases in pulmonary eosinophilia, but not to the extent that anti-B7-2 treatment alone did. This finding is consistent with the reports of other investigators using similar murine models of allergic airway responses (37). Harris et al. demonstrate that blockade of B7-1 with a mutant form of CTLA4Ig, which specifically blocks B7-1, significantly reduced Ag-induced tissue eosinophilia, but had no effect on blood eosinophilia or IgE levels (37). In contrast to the partial suppression of eosinophilia by anti-B7-1 Abs, B7-2 blockade virtually ablated Ag-induced increases in BAL eosinophils. This inhibition was concomitant with the suppression of both IL-4 and IL-5 BAL levels. On the other hand, blockade of B7-1 did not inhibit Ag-induced increases in either IL-4 or IL-5 levels. This effect was also not mediated via increases in IFN-γ as no increases in IFN-γ levels were observed following anti-B7-1 treatment of OVA-sensitized animals in our study. These results suggest that the partial inhibitory effect of anti-B7-1 is due to non-IL-5 mediated processes, which contribute specifically to recruitment of eosinophils into tissues such as the production of RANTES and/or eotaxin (39, 40).

As blockade of B7-2 2 wk after the initial sensitization was effective at suppressing the development of this type 2 immune response, our results sug[redacted] that B7 costimulatory molecules are required for the activation of T cells during this secondary response. Previous in vitro (41) and in vivo (22, 42) studies have suggested that B7 costimulatory molecules may not be required for effector and memory T cell activation and cytokine production. Our results suggest that B7-2 molecules play a pivotal role in triggering of the Th2 challenge immune response in this murine model of allergy, suggesting that costimulatory signals may be important at these later stages of T cell differentiation. Consistent with this hypothesis, Finck et al. (19) has recently shown that CTLA4Ig administration can markedly ameliorate disease severity in a murine model of lupus, if administered subsequent to the development of disease. Although this effect may also be attributed to the inhibition of newly recruited T cells during this chronic disease, it is unlikely that this is the case in our allergy model, given the acute effect, within 96 h, of blocking B7-2 interactions.

Consistent with our findings, the importance of B7-2 in the differentiation of Th2 cells has also been recently demonstrated in an in vivo model of experimental allergic encephalomyelitis (23). In this model, Th1 cytokine-producing cells are associated with the disease phenotype. Anti-B7-1 treatment (resulting in Th2 cell expansion) ameliorated the disease, whereas anti-B7-2 exacerbated the disease, presumably resulting in the expansion of Th1 cells. In contrast, type 2 immune responses in response to infection with the nematode parasite, *Heligmosomoides polygyrus*, require both B7-1 and B7-2 costimulation, as blockade of both B7-1 and B7-2 was required to inhibit *H. polygyrus*-induced type 2 immune responses such as increases in serum IgG1 and IgE levels, the expansion of lymph node germinal centers, elevated blood eosinophils, and increased mucosal mast cells (43). These studies indicate that either B7-1 or B7-2 ligand interactions can provide the required costimulatory signals that lead to T cell effector function during a type 2 in vivo immune response. Thus it is clear that the requirements for costimulatory molecules during immune responses are quite complex and are likely to depend on a number of factors unique to each specific type of immune response.

The distinct requirements for B7-2 costimulation observed in this model are likely due to differential expression of B7-1 and B7-2, as other investigators have recently shown that both lung macrophages (37) and lung B cells (35) express predominantly B7-2 following inhalational exposure to OVA of mice of two different genetic backgrounds. One possible explanation for this differential expression of B7 molecules is that previous studies have indicated that there are clear differences in the kinetics of expression of B7-1 and B7-2 during an immune response (44, 45). B7-2 is constitutively expressed on monocytes and rapidly up-regulated on B cells and dendritic cells following activation. In contrast, B7-1 expression is up-regulated later during activation of the immune response on each cell type. However, in the studies described above, B7 expression was examined several weeks after the initial sensitization, which should have been sufficient time for B7-1 expression to be up-regulated. However, as we have previously shown in a similar model that systemic sensitization alone is not sufficient to elicit allergic responses in the lung, the time following local lung challenge with the immunogen may be more critical (8). Alternatively, either the nature of the immunogen, the route of exposure and/or the dose of the immunogen may influence not only the type of immune response but the degree of costimulation required (46, 47).

In summary, our studies provide compelling evidence that the development of Th2 cytokine patterns and subsequent development of allergic airway responses to inhaled Ag challenge requires B7-2 costimulation. As a predominant Th2 cytokine pattern has been observed in human asthmatics (1–3), these studies suggest

that blockade of B7-2 may provide a novel therapeutic approach to the treatment of allergic airway disorders.

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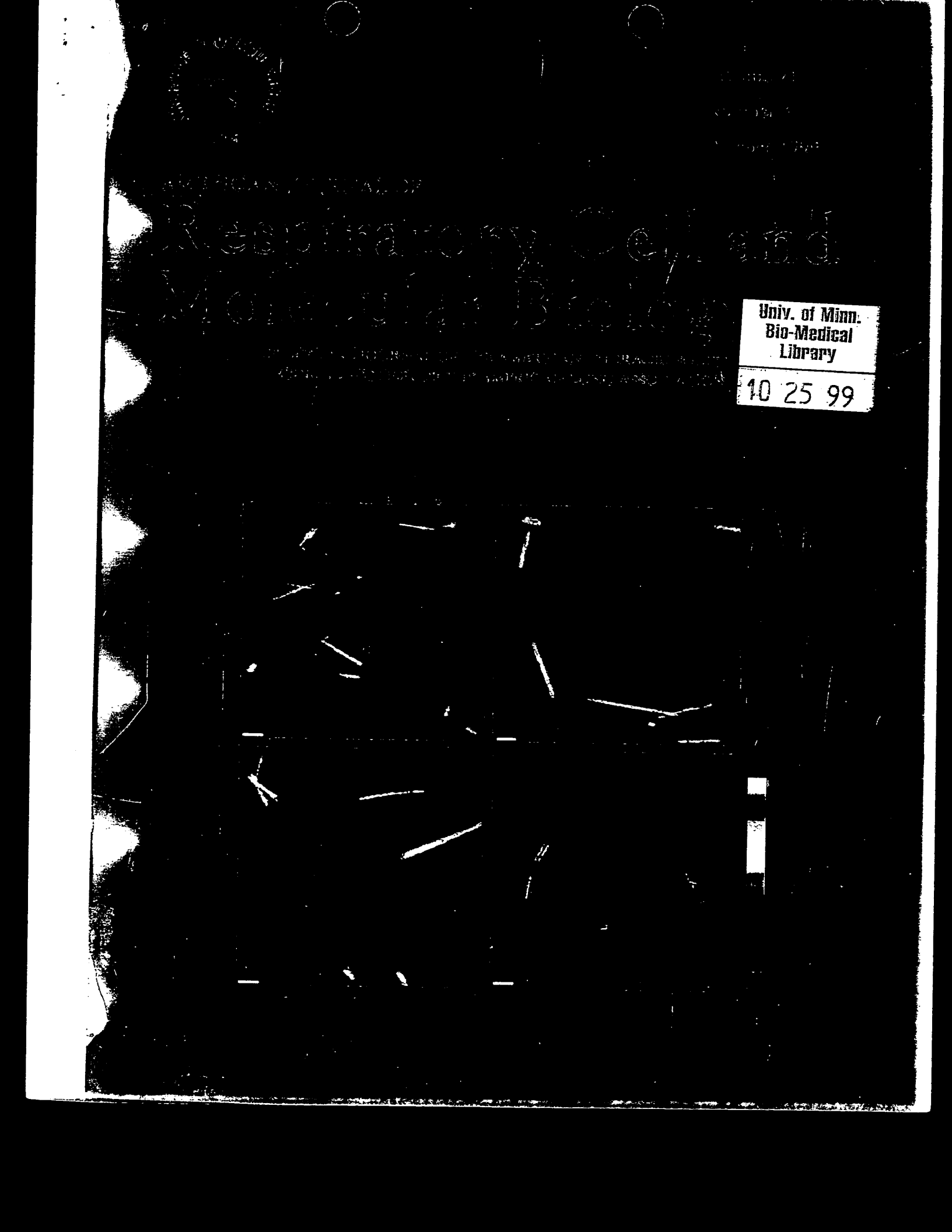
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CD28 Interactions with Either CD80 or CD86 Are Sufficient to Induce Allergic Airway Inflammation in Mice

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Previous studies have shown that the pan CD28/cytotoxic T lymphocyte antigen (CTL)A-4 antagonist CTLA4 immunoglobulin (Ig) inhibits eosinophilic airway inflammation in *Schistosoma mansoni*–sensitized and airway-challenged mice. In the present study, the importance of CD28 as well as the individual roles of CD80 and CD86 were examined in this system using wild-type and CD28 knockout (KO) mice. Unlike wild-type controls, CD28KO mice did not produce systemic IgE or eosinophilic airway inflammation after antigen challenge. However, a lymphocytic infiltrate and continued production of interferon-γ was observed in these animals. Thus, CD28 is not essential for the initial recruitment of lymphocytes into antigen-challenged airways but critically regulates the allergic T-helper 2 phenotype. We next determined by polymerase chain reaction and flow cytometry that CD80 and CD86 molecules are constitutively expressed in the naive murine lung and on eosinophils in the allergic lung, suggesting a potential important role for both ligands in the development of asthma. Combined anti-CD80/anti-CD86 treatment throughout the antigen challenge period fully blocked the development of allergic airways, whereas a partial reduction was observed in mice treated with either anti-CD80 or anti-CD86 antibody alone. However, only anti-CD86 blocked systemic IgE production. Therefore, signaling through either CD80 or CD86 is sufficient to generate a partial local allergic response, whereas CD86 costimulation is essential to induce systemic allergic (IgE) reactions. Finally, combined anti-B7 monoclonal antibody treatment after sensitization reduced airway eosinophilia and interleukin (IL)-4/IL-5 cytokine secretion consistent with an ongoing role for CD28/B7 interactions in the effector phase of the disease. These results emphasize the importance of differential B7 expression on different cells and in different organs on subsequent CD28/B7-mediated immune events, including the potential for CD28/B7 blockade in the treatment of atopic airway disease in people. **Mathur, M., K. Herrmann, Y. Qin, F. Gulmen, X. Li, R. Krimins, J. Weinstock, D. Elliott, J. A. Bluestone, and P. Padrid. 1999. CD28 interactions with either CD80 or CD86 are sufficient to induce allergic airway inflammation in mice. Am. J. Respir. Cell Mol. Biol. 21:498–509.**

Atopic asthma is a disorder characterized clinically by spontaneous airflow limitation and nonspecific airway hyperresponsiveness. The mechanism(s) that underlie these pathologic clinical findings includes airway inflammation with eosinophilic infiltration of airway epithelium and submucosa (1). The generation of eosinophilic airway inflammation appears to be dependent on cytokine signals delivered by activated T lymphocytes (2–4). It has been established that full activation of T lymphocytes to direct the activation and migration of airway eosinophils requires two signals. The first signal occurs following the interaction of polymorphic T-cell receptors with the major histocompatibility complex peptide complex on potent antigen-presenting cells (APCs), including B cells, macrophages, and dendritic cells. This engagement initiates a cascade of biochemical signals to initiate the transcription of important effector proteins such as interleukin (IL)-2 and inflammatory cytokines such as interferon (IFN)-γ, and the development of cytolytic effector molecules. Complete T-cell activation and differen-

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Abbreviations: antigen-presenting cell, APC; bronchoalveolar lavage fluid, BALF; CD28-deficient, CD28KO; cytotoxic T lymphocyte antigen, CTL; diaminobenzidine tetrahydrochloride, DAB; experimental autoimmune encephalitis, EAE; enzyme-linked immunosorbent assay, ELISA; fluorescence-activated cell sorter, FACS; fluorescein isothiocyanate, FITC; hematoxylin and eosin, H&E; interferon, IFN; immunoglobulin, Ig; interleukin, IL; knockout, KO; monoclonal antibody, mAb; median fluorescence intensity, MFI; messenger RNA, mRNA; polymerase chain reaction, PCR; sensitized and challenged, SCH; soluble egg antigen, SEA; T-helper, Th.

tiation, however, requires a second, noncognate set of interactions, the so-called costimulatory stimulus. The best-studied costimulatory stimulus involves the CD28/B7 family of molecules. CD28 is a cell-surface glycoprotein homodimer expressed on the majority of functional T cells. Both CD28 and the homologous family member cytotoxic T lymphocyte antigen (CTLA)-4 bind to the same B7 family of coreceptors, of which there are at least two members, CD80 and CD86 (5). These B7 molecules are differentially expressed on distinct tissues, have different requirements for induction, and exist in multiple forms with varying affinities for the CD28 and CTLA-4 receptors. Previous studies have shown that CD28 ligation regulates cell-cycle progression, cell survival, and cytokine and chemokine production (5).

Recently, treatment of mice with a soluble CD28/CTLA-4 antagonist, CTLA4 immunoglobulin (Ig) (a chimeric fusion protein consisting of the extracellular domain of CTLA-4 and the hinge, CH2, and CH3 regions of IgG), has been shown to inhibit the development of pathologic changes in airway structure and function in a number of murine models of asthma (6–9). We have shown that administration of CTLA4Ig after primary immunization with *Schistosoma mansoni* antigen can cause immune deviation from a primarily T-helper (Th)2–like response toward a primarily Th1-like response (8). Because CTLA4Ig inhibits the interaction of both CD28 and CTLA-4 to both CD80 and CD86, the functionally relevant interaction (i.e., CD28 or CTLA-4) and the specific roles of individual B7 molecules in the induction of T lymphocyte–mediated asthmatic airway inflammation remain unclear. In particular, the importance of CD28 in generating lymphocyte effector functions has recently been challenged by studies in CD28 "knockout" (KO) mice that are nevertheless capable of rejecting transplanted cardiac allografts in a T cell–specific manner (10, 11). Additionally, a number of cell types within the allergic lung can express B7 molecules, including T and B lymphocytes, macrophages, dendritic cells, epithelial cells, and eosinophils. However, it is not clear whether one or a combination of these cells plays a dominant role in B7 presentation in the allergic lung. An independent role for CD28/CD80 or CD28/CD86 ligation in generating the allergic airway phenotype has not been conclusively demonstrated. In the present study, we compared the effect of *S. mansoni* sensitization and challenge in wild-type mice, CD28KO mice, and mice treated with monoclonal antibodies (mAbs) to CD80 and CD86 during the entire antigen sensitization and challenge period or only during the period of antigen challenge. These studies suggest that CD28, not CTLA-4, is the essential B7 receptor for the induction and progression of Th2-mediated allergic airway inflammation in this murine model. Moreover, unlike peripheral T-cell responses, including those that control IgE production and graft rejection, CD80 and CD86 ligation provides equivalent critical costimulatory signals in response to the allergen. These results are consistent with the constitutive expression and kinetics of expression of CD80 and CD86 locally within lung in this animal model. This may be due to continued antigen stimulation in this tissue, including preferential expression of CD80 on eosinophils, a cell type that does not play a role in presentation of costimulatory molecules in nonallergic tissues. Finally, the ability of the combination of anti-CD80 and anti-CD86 mAb treatment to diminish allergic airway disease in mice after antigen sensitization suggests a potential therapeutic role for this treatment in humans with atopic asthma.

Materials and Methods

Animals

Female C57BL/6 (B6) mice (6 to 10 wk old) were purchased from Harlan Sprague-Dawley and housed in a specific pathogen–free facility maintained by the University of Chicago Animal Resources Center. CD28-deficient (CD28KO) mice were generated as previously described (12) and were bred onto a B6 background. The studies reported here conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Antibodies

Hamster-antimouse CD80 (13) and rat-antimouse CD86 (14) antibodies were produced at the University of Chicago in a high-density bioreactor (Endotronics Corp., Coons River, MN). Antibodies were purified as previously described (14, 15). Both antibodies were analyzed for binding specificity based on staining of CD80 and CD86 transfectants, respectively. As described later, mice were injected with either or both mAbs (50 μg/mouse, intraperitoneally) every other day beginning on Day 0 and throughout the treatment period. Control animals were treated with the relevant isotype control Ig (hamster or rat IgG; Southern Biotech, Birmingham, AL). Mouse CTLA4Ig was a generous gift from Genetics Institute (Cambridge, MA). CTLA4Ig was administered to a subset of CD28KO mice (50 μg/mouse, intraperitoneally) every other day beginning on Day 0 and throughout the treatment period. Anti-CD3 (145-2C11) was produced as previously described (16) and purified by passage over a protein A–coupled sepharose column. Anti-CD25, CD44, CD45, CD69, B220, and Thy1.2 used in fluorescence-activated cell sorter (FACS) analyses were obtained from PharMingen (San Diego, CA).

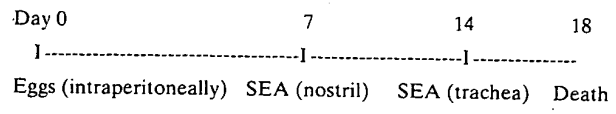
S. mansoni Eggs and Antigen

S. mansoni eggs were isolated and purified and soluble egg antigen (SEA) was produced as previously described (17). Eggs were stored at −70°C in 1.7% saline before use.

Antigen Sensitization and Challenge

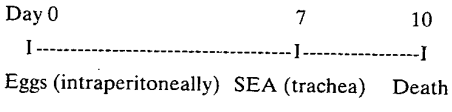
Two protocols were used in the following studies. In the first protocol, mice were immunized intraperitoneally with 5,000 isolated *S. mansoni* eggs at on Day 0. On Days 7 and 14 mice received 10 μg of SEA intranasally and intratracheally, respectively. Control animals were sensitized and challenged in the same manner with saline instead of eggs and SEA. Animals were killed 4 d after the intratracheal injection of SEA.

Protocol #1



As previously described, this protocol consistently and reliably induces maximal airway tissue eosinophilia, airway hyperresponsiveness to methacholine, and secretion of reproducible amounts of IL-5 into bronchoalveolar lavage fluid (BALF) (8). However, utilization of two airway challenges in Protocol #1 made it more difficult to evaluate the effect(s) of anti-B7 treatment in systemically sensitized mice before local antigen challenge. Therefore, Protocol #1 was modified so that airway inflammation was induced following only a single airway antigen challenge. In this second protocol, 5,000 eggs were injected intraperitoneally at Day 0 and 10 μg of SEA was injected intratracheally at Day 7. Control animals were sensitized and challenged in the same way with saline instead of eggs and SEA. Animals were killed 3 d after the intratracheal injection of SEA.

Protocol #2



Instrumentation

Mice were anesthetized by intraperitoneal injection of ketamine HCl and xylazine HCl. The trachea was cannulated with a 20-gauge, 1-cm metal needle, and the jugular vein was cannulated with P-10 tubing.

BAL

BAL was performed by injecting 0.8 ml of ice-cold phosphate-buffered saline (PBS) through the tracheal cannula and following it with gentle aspiration. This procedure was repeated three additional times. Fluids from all four lavages were pooled for maximum cell recovery. Cells were stained with trypan blue to determine viability, and total nucleated cells counts were determined using a Neubauer hemocytometer. Cytocentrifuge preparations were made using a cytocentrifuge (Shandon Southern Instruments, Sewickley, PA) set for 700 × *g* for 5 min. Cytospin slides were fixed and stained using Diff-Quik (American Scientific Products, McGaw Park, IL). Differential cell counts were determined by counting a minimum of 300 cells/slide, using standard morphologic criteria. Whole blood was withdrawn from the jugular catheter for determination of IgE.

Isolation and Stimulation of Lung Lymphocytes

Lungs from individual mice were digested for approximately 1 h in a buffer solution containing 850 U/ml hyaluronidase, 500 U/ml DNase I, and 1 mg/ml collagenase. Undigested tissue was allowed to settle and the resulting slurry was passed through a 55-μm Nytex filter. Erythrocytes were lysed and the remaining cells were washed three times in RPMI 1640 with 10% fetal calf serum (FCS). These washed cells were subsequently overlaid onto a Percoll gradient (50 to 70%). Cells within the 50 to 70% interface were aspirated and washed in complete media. An aliquot of these cells was subsequently stained with an anti-CD3 (145-2C11) mAb and analyzed by FACS to determine relative and absolute numbers of lymphocytes.

Proliferation Assay

T cells were separated from whole lung homogenates harvested from sensitized and challenged (SCH) CD28KO mice (including a group treated with CTLA4Ig) using magnetic cell sorting techniques (MACS; Miltenyi Corp., Gladbach, Germany). Cells were cultured in 96-well flat-bottomed plates coated with anti-CD3 (145-2C11, 1.0 μg/ml) or SEA (10 μg/ml) using 2×10^5 cells/well plus an equal number of irradiated spleen cells (final concentration: 2×10^6 cells/ml) in Dulbecco's modified Eagle's medium containing 10% FCS, 2 mM glutamine, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and gentamicin sulfate (5 μg/ml) at 37°C in 5% CO_2. Cells were pulsed 18 h before harvest with 1 μCi (methyl-[^{3}H]thymidine) (37 kBq; DuPont, Boston, MA) per well. The cells were harvested onto filters and the radioactivity on the dried filters was measured in a liquid scintillation counter. Incorporation during the last 18 h of culture (counts per minute) was used as an index of proliferation.

Reverse Transcriptase/Polymerase Chain Reaction Detection of CD80 and CD86 Messenger RNA Expression in Murine Lung Tissue

Many cells within murine lungs can potentially process and/or present antigen, including "professional" APCs (B cells, alveolar macrophages, dendritic cells) and nontraditional APCs (epithelial cells, eosinophils). Importantly, it has not yet been determined which one or what combination of these APCs plays a dominant role in T-lymphocyte activation in human asthma or in mouse models of asthma. Therefore, we evaluated the relative expression of messenger RNA (mRNA) for both CD80 and CD86 from all collagenase-digested lung cells at baseline, after sensitization but before challenge and at 12, 24, 48, and 72 h after SEA challenge in sensitized mice. Total RNA was isolated from 100 mg lung tissue from naive and sensitized mice using standard methodology (RNA "STAT-60" reagent; Tel-Test Inc., Friendswood, TX). RNA was treated with DNase I as follows: 50 μg RNA, 3 μl 10× DNase I digestion buffer, and 3 μl DNase I (1 U/μl; GIBCO BRL, Grand Island, NY), with diethylpyrocarbonate H_2O to a total volume of 30 μl, incubated at room temperature for 10 min. The reaction was stopped by adding 3 μl 0.5 mM ethylenediaminetetraacetic acid, followed by a 10-min incubation at 65°C. Samples were purified by phenol:chloroform:isoamyl alcohol extraction.

A total of 1 μg of RNA from each of the time points was transcribed by using 1 μg of MuLV Reverse Transcriptase in a total volume of 20 μl at 42°C for 15 min, followed by polymerase chain reaction (PCR) amplification. PCR reaction conditions were as follows: 94°C/15 s, 57°C/15 s, and 72°C/30 s, followed by a final extension step at 72°C/7 min.

The primers used in PCR were β-actin: 5′ primer 5′-ACCAGGGTGTGATGGTGGGAATGGG-3′, 3′ primer 5′-TTGCTGATCCACATCTGCTGGAAGG-3′; CD80: 5′ primer 5′-TGCTGTCTGTCATTGCTGGGAAACT-3′,

3′ primer 5′-CCCAGGTGAAGTCCTCTGACACGTG-3′; and CD86: 5′ primer 5′-TCCAGAACTTACGGAAG-CACCCACG-3′, 3′ primer 5′-CAGGTTCACTGAAGT-TGGCGATCAC-3′.

A total of 15 μl of each PCR product was electrophoresed on a 2% agarose gel without loading buffer. The gel was then stained in ethidium bromide media for 20 min, soaked in distilled H_2O for 10 min, and photographed with a Kodak DC120 digital camera under ultraviolet light. The digital images were analyzed with Kodak Digital Science 1D image analysis software (Kodak, Rochester, NY) and the net intensity of each band was determined. The relative intensity of β-actin complementary DNA was detected as a positive control for each mRNA sample.

Surface Expression of B7 Molecules on Lung Cells

Cell-surface expression of CD80 and CD86 on collagenase-digested murine lung cells at equivalent time points was determined as described for mRNA studies. Cells were washed with standard FACS buffer (PBS, 0.1% sodium azide, and 0.5% bovine serum albumin, pH 7) and incubated with mAbs specific for CD80 or CD86 (1:160 dilution for both mAbs) at a final concentration of 10^5 cells/well. Propidium iodide staining was performed to exclude dead cells from analysis. A total of 5 to 10 × 10^3 cells was analyzed for each monoclonal antibody using Lysis II software. Discrete cell populations identified in scatter plots were sorted, cytocentrifuged, and deposited on glass slides (Shandon Southern Instruments), stained with Diff-Quik or Wright stain, and identified based on typical morphologic criteria and staining characteristics. Eosinophils were specifically identified on the basis of the presence of discrete eosin-positive stained granules.

Enzyme-Linked Immunosorbent Assay for Determination of IL-4, IL-5, IFN-γ, and IgE

Cytokines in BALF (IL-4, IL-5, and IFN-γ) and IgE in serum were detected by commercially available enzyme-linked immunosorbent assay (ELISA) kits (IFN-γ and IL-4: Endogen, Cambridge, MA; IL-5 and IgE: PharMingen). The lower limits of detection for IL-4, IL-5, IFN-γ, and IgE were 10, 10, and 160 pg/ml, and 167 ng/ml, respectively.

Histology

Lungs from mice randomly chosen from all groups were removed from the chest cavity and fixed by injection of 10% buffered formalin (1.0 ml) into the tracheal cannula at a pressure of 20 cm H_2O, and immersed in formalin for 24 h. All lobes were sectioned sagittally, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E) for routine analysis.

Statistical Methods

Differences between groups for BAL eosinophils, cytokine content in BALF and lung lymphocyte culture supernatant, and IgE were determined by analysis of variance. Statistical analyses for all tests were performed using a single value for each animal. All data are expressed as means ± standard error (SE). The number of animals per group was determined by power analysis using the following parameters: $\alpha = 0.05$, difference between groups = 50%, power = 0.8 (18).

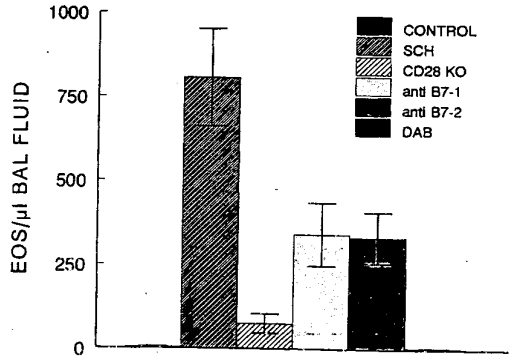


Figure 1. CD28/B7 interactions are required to generate eosinophil inflammation in *S. mansoni*–sensitized and airway-challenged mice. BAL was performed through a previously placed endotracheal tube. Four 0.8-ml aliquots of normal saline were infused, gently aspirated, and pooled. No eosinophils were recovered in BALF from the control group. Antigen challenge in *S. mansoni*–sensitized mice (SCH) resulted in dramatic BAL eosinophilia (807 ± 145 eos/μl BALF), which was profoundly inhibited in CD28KO mice (75 ± 29 eos/μl BALF, $P < 0.005$ versus SCH) or wild-type mice treated with anti-CD80 and anti-CD86 mAbs (DAB; 5 ± 2 eos/μl BALF, $P < 0.001$ versus SCH) beginning at antigen sensitization. Treatment with either anti-CD80 or anti-CD86 reduced BAL eosinophilia by 60% ($P < 0.05$ versus SCH for anti-CD80 or anti-B72). *Bars* represent the mean and SE of each group.

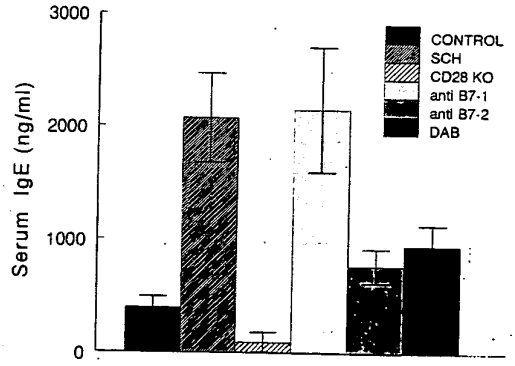


Figure 2. Anti-CD86 but not anti-CD80 treatment inhibits the systemic production of IgE. Serum was analyzed for IgE content by ELISA. IgE serum levels in SCH mice were increased almost 6-fold compared with CONTROL values (2,074 ± 394 ng/ml serum SCH versus 399 ± 94 ng/ml serum CONTROL). This was virtually abolished in CD28KO animals (92 ± 88 ng/ml serum, $P < 0.01$ versus SCH). Treatment with anti-CD86 significantly inhibited IgE production (768 ± 150 ng/ml serum, $P < 0.05$ versus SCH), and was equivalent to the effect produced by combined antibody therapy (double antibody [DAB], 950 ± 179 ng/ml serum, $P < 0.01$ versus SCH). In contrast, anti-CD80 treatment had no effect (2,148 ± 553 ng/ml serum).

Results

Effect of CD28 Gene Disruption on the Development of Atopic Airway Inflammation after *S. mansoni* Sensitization and Challenge

We have previously shown that mice sensitized and challenged with *S. mansoni* developed eosinophilic airway inflammation and airway hyperresponsiveness, and a Th2-like pattern of cytokine secretion; increased IL-5 and decreased IFN-γ in BALF, and increased IL-4 secreted from cultured lung lymphocytes *in vitro* (8). Administration of CTLA4Ig at a time of systemic antigen challenge prevented the development of the full allergic phenotype in this model, in part by causing immune deviation from a Th2-like cytokine response toward a Th1-like response. These earlier data, however, could not distinguish between the effects of CTLA4Ig in blocking CD28 versus CTLA-4 interactions. Further, we considered the possibility that CTLA4Ig might act directly on APCs through binding and crosslinking the B7 molecules (19). Previous studies from our group have shown that T cells isolated from CD28KO mice demonstrate similar abnormalities *in vitro* as do T cells stimulated in the presence of CTLA4Ig. T-cell proliferation, IL-2 production, and cell survival are diminished and, most significantly, Th2 responses are compromised (20). Therefore, CD28KO mice were examined in this *in vivo* model of atopic airway disease. As seen in Figure 1, a large number of eosinophils were found in BALF from SCH mice (807 ± 145 eos/μl BALF). In contrast, BAL eosinophilia in CD28KO mice (75 ± 29 eos/μl BALF, $P < 0.005$

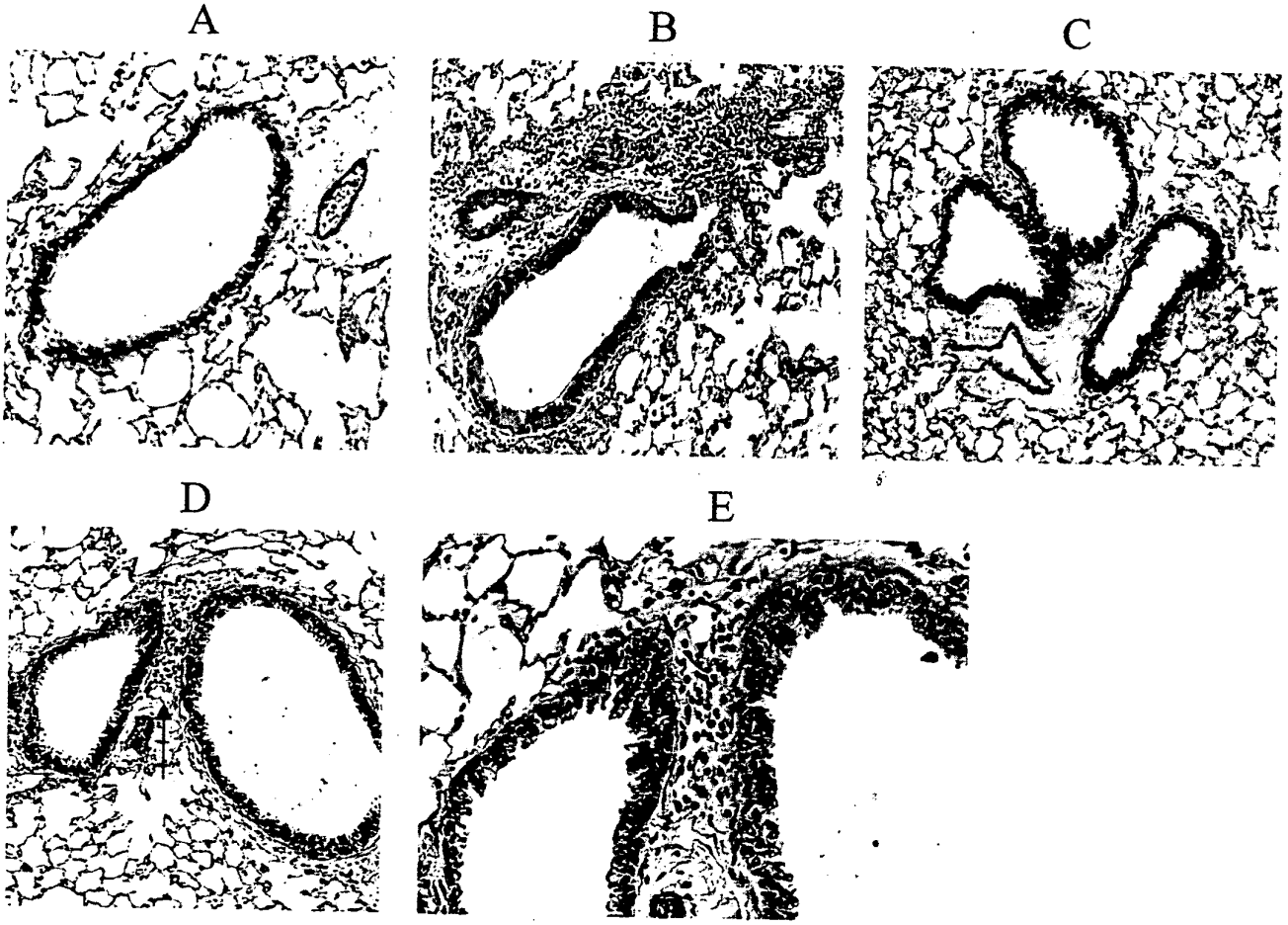


Figure 3. CD28KO mice developed mild airway lymphocytosis after antigen challenge with *S. mansoni*. Lungs from mice from all groups were fixed *in situ* with 10% formalin for 24 h and stained with H&E for routine analysis. (*A*) Lung from control mouse. There is no inflammatory infiltrate or edema around bronchioles. H&E stain, original magnification: ×400. (*B*) Lung from SCH mouse. There is a marked, predominantly eosinophilic infiltration and edema around bronchioles and vessels, with extension into the adjacent alveolar parenchyma. H&E stain, original magnification: ×400. (*C*) Lung from mouse treated with both anti-CD80 and anti-CD86 mAbs. There is minimal inflammation within and surrounding bronchioles and vessels. An occasional eosinophil is seen. H&E stain, original magnification: ×200. (*D*) Lung from CD28KO mouse. There is a mild peribronchiolar and perivascular lymphocytic infiltrate (*arrow*). Eosinophilic infiltration is not seen. H&E stain, original magnification: ×400. (*E*) High-power view of cellular infiltrate from *D*. H&E stain, original magnification: ×600.

TABLE 1
Effect of CTLA4Ig treatment on CD28KO mice sensitized and challenged with S. mansoni

	Wild-Type SCH ($n = 5$)	Wild-Type SCH + CTLA4Ig ($n = 5$)	CD28KO Control ($n = 3$)	CD28KO SCH ($n = 6$)	CD28KO SCH + CTLA4Ig ($n = 6$)
Eosinophils, % (BALF)	83 ± 1	49 ± 13	0	17 ± 4	15 ± 6
Total number of eosinophils (μl BALF)	331 ± 115	83 ± 38	0	9 ± 4	9 ± 4
Lymphocytes, % (BALF)	7 ± 2	12 ± 5	< 5	30 ± 6	17 ± 3
Total number of lymphocytes (μl BALF)	28 ± 6	20 ± 6	< 2	17 ± 7	10 ± 3
IL-4 (pg/ml BALF)	17 ± 1	13 ± 1	< 10	< 10	< 10
IL-5 (pg/ml BALF)	162 ± 24	59 ± 15	< 10	< 10	< 10
IFN-γ (ng/ml BALF)	1.15 ± 0.12	0.61 ± 0.09	0.64	0.91	0.83
Serum IgE (ng/ml)	3,447 ± 387	473 ± 158	ND	254 ± 21	257 ± 34
Proliferation (CPM; 2C11)	ND	ND	ND	14,588 ± 2,307	13,129 ± 2,130
Proliferation (CPM; SEA)	ND	ND	ND	8,495 ± 659	9,801 ± 880
(% cells) Thy 1.2+ CD25+	ND	ND	2.3 ± 0.2	5.5 ± 0.8	5.0 ± 0.2
(% cells) Thy 1.2+ CD69+	ND	ND	3.6 ± 0.7	6.2 ± 1.2	4.8 ± 0.4
(% cells) Thy 1.2+ CD44+	ND	ND	53.3 ± 1.9	54.0 ± 1.8	57.2 ± 2.9

ND: not determined.

versus wild type) was reduced by greater than 90%. Systemic IgE production was abolished in the CD28KO animals as well (2,074 ± 394 ng/ml IgE wild type versus 92 ± 88 ng/ml IgE for CD28KO, $P < 0.001$, Figure 2). However, the immune response was not totally ablated in these animals because a prominent population of lymphocytes was observed in the BALF. Although the total number of lymphocytes in BALF was not statistically significantly different, lymphocytes accounted for 30 ± 6% of all BAL cells in the CD28KO SCH group, compared with < 7% of BAL cells in isotype-treated CD28-KO or wild-type mice ($P < 0.05$; Table 1). These findings were confirmed histologically (Figures 3A–3D). Airways from SCH mice had consistent pathologic changes in airway epithelium and submucosa. Specifically, eosinophilic infiltration within epithelium and lamina propria and goblet-cell hyperplasia were never seen in control airways (Figure 3A) but were observed in many airways from all SCH mice (Figure 3B). Eosinophilic infiltration and mucus-cell hyperplasia were not seen in the CD28KO animals; however, there was a mild peribronchiolar lymphocytic infiltrate (Figures 3D and 3E). Thus, although SCH induced CD28-deficient mice to generate a lymphocytic infiltrate, there were no additional abnormal airway changes, including epithelial derangement or increases in goblet cells or submucosal glands.

The absence of eosinophils but presence of lymphocytes in the CD28KO mice suggested a significant alteration of Th1/Th2 balance in these sensitized and challenged mice. To access the cytokine milieu directly, BALF was extracted and evaluated for the presence of IL-5 and IFN-γ. The volume of recovered BALF was equivalent between groups and ranged from 2.5 to 2.8 ml. Unlike the BALF from control sensitized and challenged mice, the BALF from SCH CD28KO mice did not contain (Th2-derived) IL-5 in response to antigen challenge (159 ± 25 pg/ml IL-5 BALF wild type versus < 10 pg/ml IL-5 BALF for CD28KO; Figure 4A). The absence of IL-5 might explain the reduction of eosinophilia in the CD28KO mice, although additional factors—including a reduction in IL-4 and the possible inhibition of chemokine-directed eosinophilotaxis—may play significant roles as well. Interestingly, secretion of the prototype Th1 cytokine (IFN-γ) in BALF from CD28KO animals was equal to wild-type mice (0.96 ± 0.2 ng/ml IFN-γ BALF CD28KO versus 0.91 ± 0.11 ng/ml IFN-γ BALF wild type, Figure 4B). Thus, our data support an essential role for CD28 signaling in the development of eosinophilia and production of Th2-type cytokines by the infiltrating BAL T cells.

Effects of Anti-CD80 and Anti-CD86 Treatment in the Development of Allergic Airway Inflammation

Mice were treated from the outset with a combination of both anti-CD80 and anti-CD86 mAbs. As seen in Figure 1, there were virtually no eosinophils (5 eos/μl) in BALF in mice treated with both anti-B7 mAbs. Cytokine analysis of the BALF confirmed the suppression of the Th2 responses in mAb-treated mice. Little IL-5 in BALF (25 ± 10 pg/ml versus 159 ± 25 pg/ml for SCH mice) was observed in treated animals (Figure 4A). IFN-γ production was also greatly reduced (0.211 ± 0.04 ng/ml versus 0.91 ± 0.11 ng/ml SCH, $P < 0.005$) in BALF from mice treated with both mAbs (Figure 4B). Finally, combined antibody treatment greatly decreased serum IgE responses (950 ± 179 ng/ml versus 2,074 ± 394 ng/ml for SCH, $P < 0.01$, Figure 2) and blocked all histologic evidence of the disease (Figure 3C).

Most *in vivo* studies have suggested that CD86 is critical for initiating immune responses and is essential for Th2 development (5). However, CD80 has been found to play an important role in the late stages of some Th2-mediated disorders, including autoimmune disease (21). The role of CD80 and CD86 in asthma is also controversial. For example, Harris and colleagues determined that CD80 ligation was critical for development of allergic airway inflammation (22). In contrast, other studies have suggested that CD86 plays a dominant role in the development of murine asthma (23, 24). Therefore, we examined the effects of anti-B7 mAbs individually beginning at the time of antigen sensitization. Treatment with either anti-CD80 or anti-

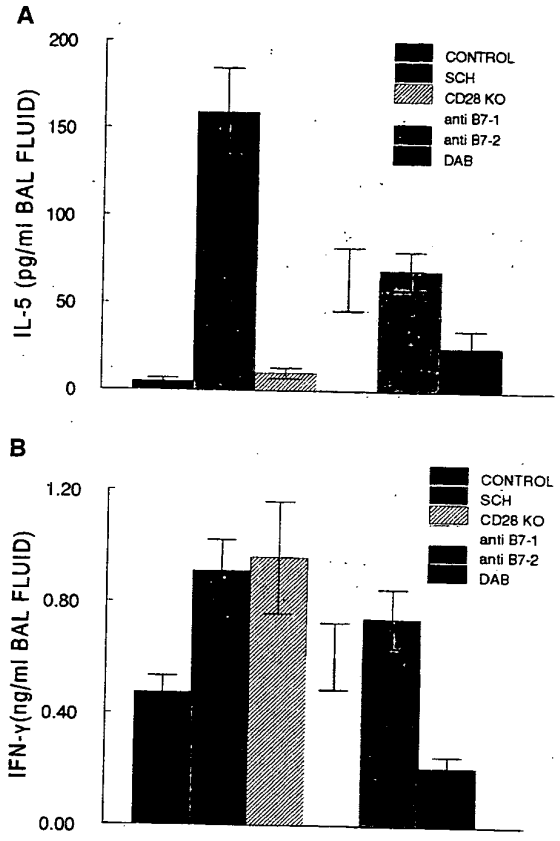


Figure 4. (*A*) IL-5 production is dramatically reduced in CD28-KO mice and wild-type mice treated with anti-B7 antibodies. Cell-free BALF was evaluated for IL-5 and IFN-γ content, respectively, by ELISA. CONTROL mice produced minimal IL-5 (5 ± 1 pg/ml BALF). In contrast, SCH mice produced large amounts of this cytokine (159 ± 25 pg IL-5/ml BALF). Secretion of IL-5 was abolished in CD28KO mice (10 ± 3 pg IL-5/ml BALF, $P < 0.001$ versus SCH) and was dramatically inhibited in animals treated with both anti-B7 antibodies (25 ± 10 pg IL-5/ml BALF, $P < 0.005$ versus SCH). Either anti-CD80 or anti-CD86 antibody treatment inhibited secretion of this "Th-2–like" cytokine by > 60% (64 ± 18 pg/ml BALF and 69 ± 11 pg/ml BALF, respectively, $P < 0.05$ versus SCH). (*B*) IFN-γ production was not affected by genetic deletion of CD28. CD28KO and wild-type mice secreted equivalent amounts of IFN-γ into BALF (0.96 ± 0.2 ng/ml BALF, CD28KO; 0.91 ± 0.11 ng/ml BALF, SCH). Treatment with individual anti-B7 antibodies had no significant effect on BALF IFN-γ content (0.61 ± 0.24 ng/ml BALF, anti-CD80; 0.74 ± 0.15 ng/ml BALF, anti-CD86). However, treatment with both antibodies (DAB) reduced BALF IFN-γ content by 75% (0.211 ± 0.04 ng/ml BALF, $P < 0.005$ versus SCH).

CD86 mAb from Day 0 resulted in significantly reduced airway eosinophilia (Figure 1) (341 ± 94 eos/μl BALF anti-CD80–treated mice, $P < 0.01$ versus SCH; 332 ± 76 eos/μl BALF anti-CD86–treated mice, $P < 0.01$ versus SCH). These findings were confirmed by histologic examination revealing that treatment with either anti-B7 mAb reduced but did not abolish airway tissue eosinophilia. Inhibition of either CD80 or CD86 ligation also reduced, but did not completely inhibit, secretion of IL-5 (64 ± 18 pg/ml BALF and 69 ± 11 pg/ml BALF, respectively, $P < 0.05$ versus SCH; Figure 4A). Interestingly, anti-CD86 but not anti-CD80 antibody treatment attenuated production of IgE in serum (768 ± 150 ng/ml BALF and 2,148 ± 553 ng/ml BALF, respectively, versus 2,074 ± 394 ng/ml BALF for SCH group, $P < 0.05$ versus anti-CD86–treated group, Figure 2). Moreover, the level of IgE suppression was equivalent to that observed in the group treated with the combination of anti-CD80 and anti-CD86 mAbs. Finally, IFN-γ secretion into BALF was not significantly affected by treatment with antibody against either CD80 or CD86 (Figure 4B). Together, these results suggest that the ligation of CD28 with either B7 molecule is sufficient to induce a partial local Th2-type cytokine response. However, CD86 ligation, not CD80, is essential for the induction of a systemic IgE response.

Effects of CTLA4Ig Treatment on *S. mansoni*–Sensitized and Challenged CD28KO Mice

In contrast to animals treated with both anti-B7 antibodies, the CD28KO mice generated significant IFN-γ in BALF, and developed mild lymphocytic inflammation of airways. Therefore, to test the possibility that an alternative T-cell surface molecule may interact with B7 to costimulate this lymphocytic response, we treated an additional six CD-28KO mice with CTLA4Ig, beginning at sensitization. This soluble chimeric fusion protein binds CD80 and CD86 with a 20-fold greater avidity compared with CD28 (25). Five wild-type animals were sensitized and challenged to serve as controls, and an additional five wild-type animals were treated with CTLA4Ig to confirm the efficacy of this drug to block CD28/B7 interactions. As expected, wild-type animals generated an allergic phenotype that was greatly inhibited by pretreatment with CTLA4Ig. However, in the CD-28KO mice CTLA4Ig treatment had no significant effect on airway lymphocytosis (30 ± 6% lymphs/BALF, isotype-treated versus 17 ± 3 lymphs/BALF, CTLA4Ig-treated, P = ns). Additionally, spleen cells from SCH CD28KO mice treated with CTLA4Ig or isotype control proliferated in an equivalent manner in response to 2C11 and SEA, and had similar surface expression of CD25, CD44, and CD69 (Table 1). Thus, engagement of B7 with an alternative molecule(s) including CTLA-4 could not explain the different results obtained from CD28KO and anti-CD80/anti-CD86–treated wild-type animals.

Effects of Anti-CD80 and Anti-CD86 Treatment during the Challenge Phase of Allergic Airway Inflammation

We next evaluated the effect(s) of treatment with anti-B7 antibodies when administered after antigen sensitization and during antigen challenge inasmuch as this setting might more closely approximate the clinical situation in which already-sensitized patients become symptomatic upon exposure to the sensitizing antigen. Mice were given *S. mansoni* as described and treated with either anti-CD80 or anti-CD86 or both mAbs beginning at the time of antigen challenge (daily from Days 7 to 10). Treatment with either mAb alone had no significant effect on any of the disease parameters. However, the combination of both mAbs during the challenge period resulted in a 75% reduction in airway eosinophilia and IL-4 and IL-5 production in BALF. Interest-

TABLE 2
Effect of anti-B7 mAb treatment given during antigen challenge (Days 7 to 10) in S. mansoni*–sensitized mice*

	% EOS BALF	TOTAL EOS (μl) BALF	IL-4 (pg/ml) BALF	IL-5 (pg/ml) BALF	IFN-γ (ng/ml) BALF	IgE (ng/ml) Serum
Control (n = 8)	< 1	< 1	< 3	< 5		565 ± 241
SCH (n = 15)	70 ± 3	250 ± 33	54 ± 8	186 ± 31	1.15 ± 0.08	1,586 ± 162
Anti–B7-1 Days 7 to 10 (n = 10)	69 ± 5	214 ± 45	58 ± 9	140 ± 25	1.11 ± 0.09	1,519 ± 433
Anti–B7-2 Days 7 to 10 (n = 10)	57 ± 9	135 ± 53	36 ± 6	97 ± 18	0.90 ± 0.07	1,230 ± 254
DAB Days 7 to 10 (n = 10)	42 ± 9	67 ± 28*	18 ± 6†	49 ± 13‡	0.93 ± 0.09	1,487 ± 253

EOS: eosinophils.
*P < 0.001 versus SCH.
†P = 0.03 versus SCH.
‡P = 0.02 versus SCH.

ingly, production of IFN-γ in the BALF and serum IgE levels were equivalent to those in SCH mice (Table 2).

Expression of CD80 and CD86 in SCH Mouse Lung

CD86 is generally assumed to play a dominant role early in the course of T-cell activation. However, the relative effectiveness of anti-CD80 mAb in the local immune responses in the lung, especially late in the disease progression, suggested that this costimulatory ligand might be selectively upregulated in lung tissue. Thus, we examined the expression of CD80 and CD86 mRNA in lung tissue from SCH mice, beginning at a time point from sensitization through 72 h after antigen challenge. We found that both CD80 and CD86 mRNA (not shown) were detected in the lungs of unmanipulated mice as well as in SCH mice examined after challenge with SEA. Next, surface expression of both B7 molecules on collagenase-digested lung cells was assessed by FACS analyses at equivalent time points after antigen challenge in SCH mice (Figure 5). CD80 surface expression was constitutive (median fluorescence intensity [MFI] 6 ± 1), was upregulated within 24 h (MFI = 19 ± 1), and continued to be significantly expressed at 72 h after antigen challenge (MFI = 15 ± 1). Surface expression of CD86 was also constitutive (MFI = 11 ± 2) and elevated within 24 h (MFI = 21 ± 2), although expression returned to baseline levels by 72 h after antigen challenge (MFI = 12 ± 1).

Of particular interest was the finding that eosinophils expressed primarily CD80 (26% of all cells) versus CD86 (10% of all cells; Figure 6) at 72 h. In contrast, alveolar macrophages (data not shown) and lymphocytes expressed primarily CD86 (28% of all cells), with lesser expression of CD80 (7% of all cells) at this time point. We are aware of one previous study that addressed B7 expression on eosinophils. In this study, the authors demonstrated upregulation of both CD80 and CD86 expression on eosinophils from peritoneal exudate of IL-5 transgenic mice after stimulation with granulocyte macrophage colony-stimulating factor (26). Thus our data again emphasize the potential differences in B7 expression on different cells in different immune environments. Importantly, these data also raise the possibility that eosinophils may provide additional costimulatory signals in a rapidly developing allergic environment, thus potentially making the allergic lung a somewhat unique organ in terms of expression of B7 costimulatory molecules.

Discussion

The results of the current study illustrate the central role of CD28 in the induction of allergic airway responses in *S. mansoni*–sensitized and airway-challenged mice. These animals develop an atopic respiratory phenotype including airway eosinophilia and goblet-cell hyperplasia, secrete IL-4 and IL-5 locally within the respiratory tract, and produce significant amounts of systemic IgE. Either CD28-deficient mice or combined treatment with anti-CD80 and anti-CD86 mAbs completely suppressed the Th2-driven allergic airway inflammatory response, whereas treatment with either anti-CD80 or anti-CD86 mAbs had an equivalent effect to partially suppress allergic inflammation. In addition, simultaneous treatment of sensitized mice at the time of antigen challenge with both anti-CD80 and anti-CD86 mAbs significantly inhibited the development of airway eosinophilia, histologic evidence of mucus cell hyperplasia/hypertrophy, and the secretion of IL-4 and IL-5 in BALF. We have previously reported that treatment with CTLA4Ig after antigen sensitization blocked the development of airway eosinophilia, airway hyperresponsiveness, and production of Th2-like cytokines and systemic IgE in *S. mansoni*–sensitized and challenged mice (8). Thus, our present findings suggest that blocking CD28/B7 interactions either before or during antigenic challenge inhibits the development of Th2-like lymphocytes. Because asthma is not reliably predicted in asymptomatic people, the treatment of asthmatic patients is initiated after primary immunization with antigen. Thus, our current data imply that inhibiting the T-lymphocyte costimulation pathway in sensitized individuals may be a potentially worthwhile therapeutic strategy to treat people with atopic respiratory disorders. However, our findings also suggest that strategies that are designed to inhibit CD28/B7 interactions should be based on the particular individual kinetics of expression of CD80 and CD86 in human lung tissue.

Although our results suggest that CD28 ligation is necessary for production of Th2 cells, sensitized CD28KO mice produced significant IFN-γ and developed a mild lymphocytosis in response to antigen challenge. This was in contrast to wild-type mice treated with mAbs to both B7 molecules, in which IFN-γ production and lymphocyte infiltrates were both inhibited. These data are similar to those reported by Brown and associates (27) in which T cells from CD28KO mice on either a C57BL/6 or BALB/c

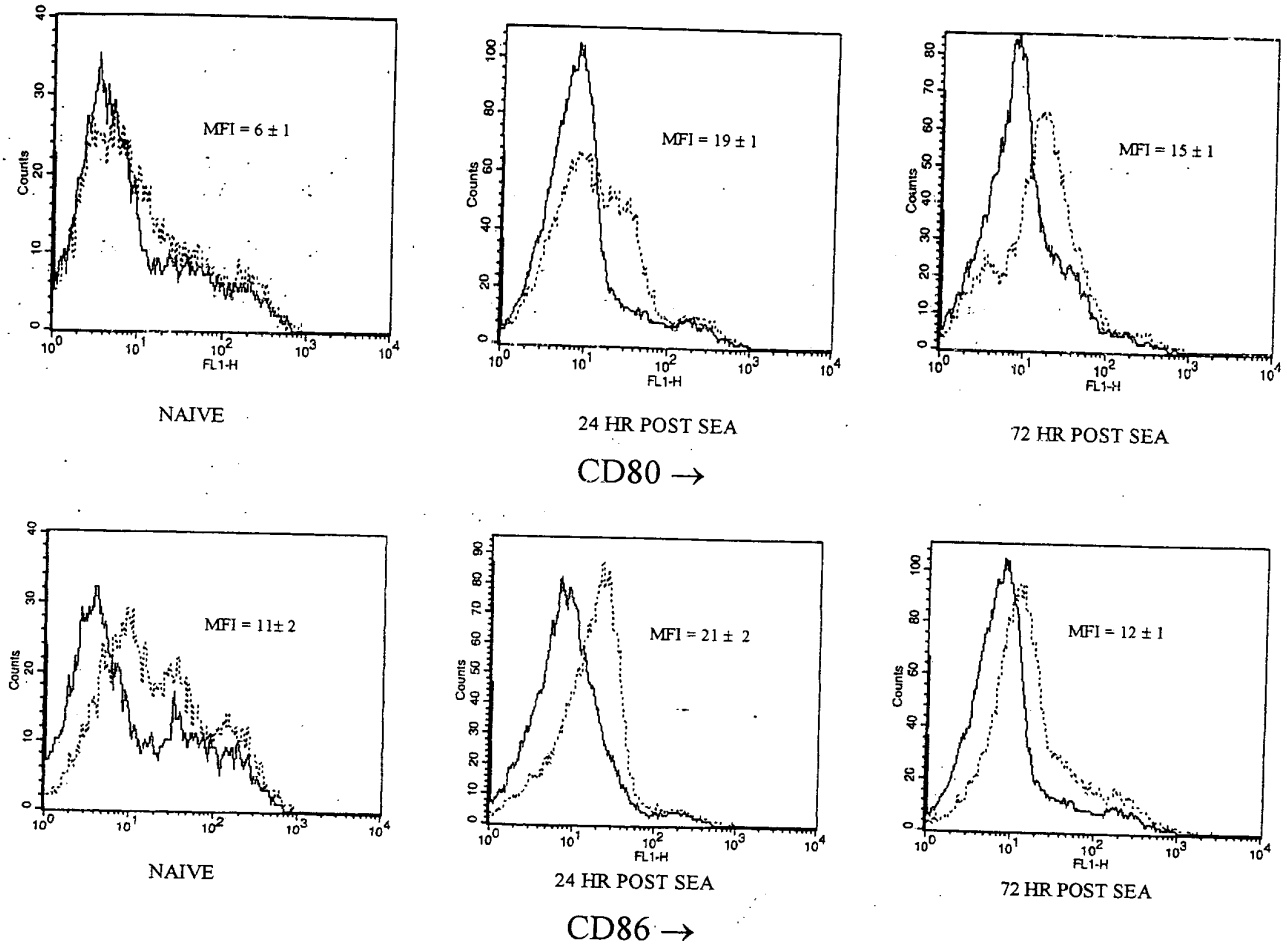


Figure 5. Representative FACS analysis of B7 expression on whole lung cells from antigen-sensitized and challenged mice. Histograms derived from a scatter plot using gated, live cells obtained by collagenase digestion of whole lung from *S. mansoni*–sensitized mice before (naive) and 24 and 72 h after antigen challenge. Histograms represent unstained cells (*solid lines*) or cells stained with anti–CD80 fluorescein isothiocyanate (FITC) (*dashed lines*, CD80) or anti–CD86 FITC (*dashed lines*, CD86) at the time points indicated. Lung cells from CD80KO and CD86KO mice were used as negative controls (not shown). Data are representative of four to seven experiments for each time point.

background produced IFN-γ in response to infection with *Leishmania major* when compared with wild-type controls. These data also support findings from our lab and others (10) demonstrating that CD28KO mice can mount an efficient, although delayed, Th1 rejection response after organ transplantation.

The explanation for these differing results obtained in the CD28KO mouse and in wild-type mice treated with anti-CD80 and anti-CD86 mAbs is not clear. We considered the possibility that there may be an additional B7-dependent costimulatory ligand(s) on T cells responsible for induction of partial T-cell activation. However, we observed that blockade of B7 ligation with CTLA4Ig did not affect the generation of IFN-γ or the development of a mild lymphocytic infiltrate in these sensitized and challenged CD28KO mice. Thus, it is more likely that the data reflect intrinsic differences between antibody treatment of the CD28/B7 pathways and gene disruption of CD28. In this regard, heart allograft rejection that is fully blocked by CTLA4Ig is not prevented in CD28KO mice even after CTLA4Ig therapy. In fact, blocking CTLA-4/B7 interactions accelerates Th1-mediated allograft rejection in CD28-deficient mice (10). Moreover, we have demonstrated that T cells from CD28KO mice expand significantly before activation-induced cell death (unlike cells cultured with CTLA4Ig [28]). We speculate that the absence of CD28 expression may result in a subtle alteration in T-cell development (29, 30) or changes in mature T-cell signal transduction due to the absence of cell surface membrane–expressed CD28 that can interact with intracellular signaling molecules such as ITK (31) or PI3 kinase (32).

Our data also raise several questions with regard to the individual role of the B7 molecules and the importance of the temporal expression of these costimulatory ligands in

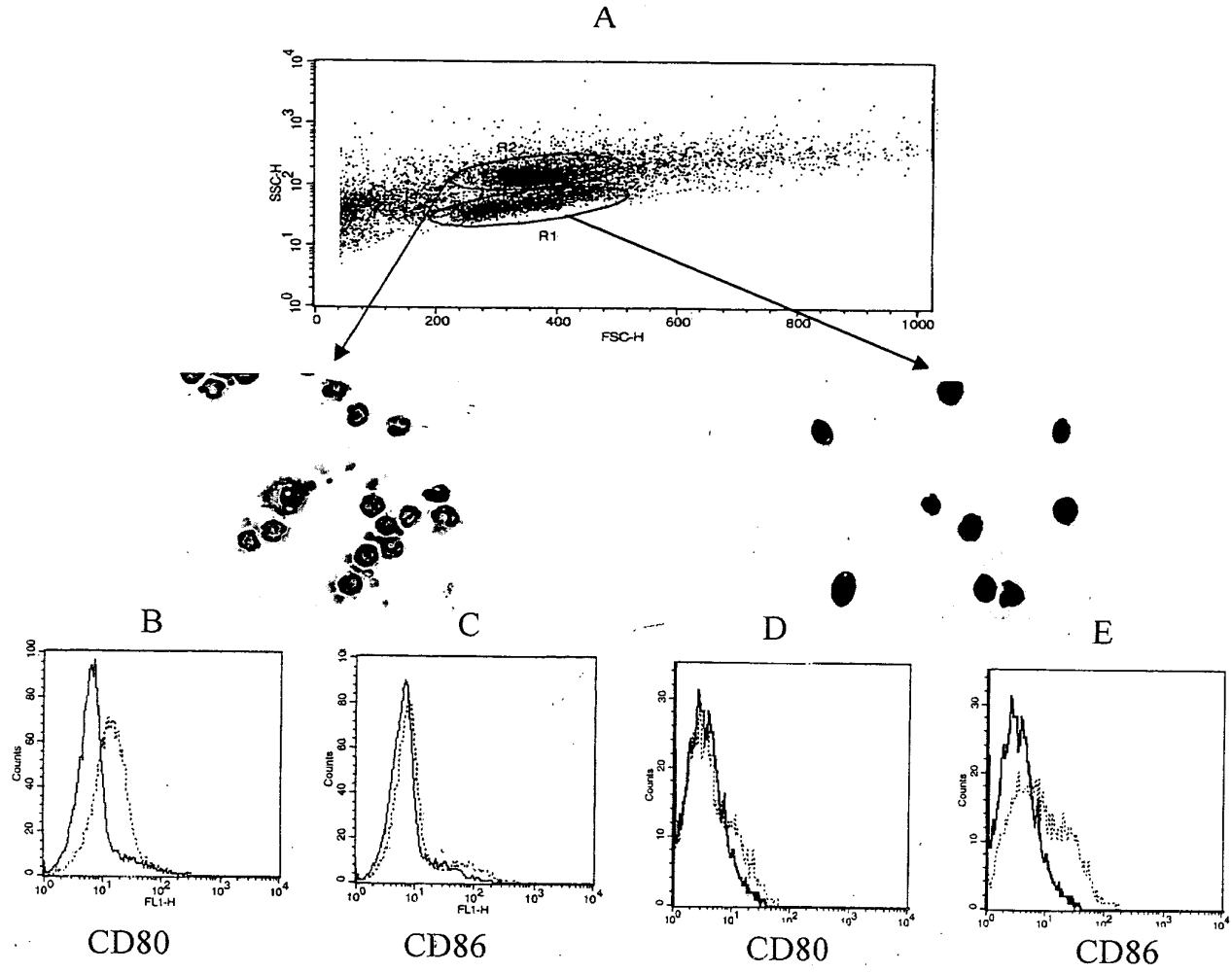


Figure 6. Flow cytometric detection of B7 expression on eosinophils and lymphocytes from lungs of antigen-sensitized and -challenged mice. Scatter plot (*A*) of cells obtained by collagenase digestion of whole lung from *S. mansoni*–sensitized mice 72 h after injection of antigen into trachea. The gated regions were sorted and contained 95% eosinophils (R2, < 1% eosinophils at baseline) or lymphocytes (R1), respectively, at 72 h. Eosinophils stained positively with FITC-conjugated anti-CD80 (*B, dashed lines*, 26% of all cells) and FITC-conjugated anti-CD86 (*C, dashed lines*, 10% of all cells) compared with FITC-conjugated control mAb (*B* and *C, solid lines*). For comparison, B7 staining on lymphocytes was somewhat different, with 7% of all cells staining positively for CD80 (*D*) versus 28% positive for CD86 (*E*). Data are representative of four to seven experiments.

promoting T-cell effector functions *in vivo*. Specifically, in the current study, antibody treatment directed against either CD80 or CD86 alone had a partial and similar inhibitory effect on the development of allergic inflammation. Although several studies have reported that CTLA4Ig can block the development of allergic airways in small-animal models, the individual roles of CD80 and CD86 have remained controversial. For example, Harris and coworkers have described the requirement for CD80 but not CD86 in the induction of murine allergic airway inflammation (22). In apparent contradiction, two additional studies reported that anti-CD86 treatment of ovalbumin (OVA)-sensitized mice immediately before antigen challenge resulted in greatly diminished airway eosinophilia and airway hyperresponsiveness (23, 24). However, in both studies eosinophilic recruitment into airways was also significantly reduced in mice treated with only the anti-CD80 antibody.

Perhaps not surprisingly, CD80 and CD86 play significantly different roles in determining immune responses *in vivo* in other, nonasthma models as well (33). For example, we and others have shown that selective inhibition of B7 ligands can alter the disease state in an animal model of diabetes. Non-obese diabetic mice treated with anti-CD86 antibodies had a decreased incidence of diabetes, whereas treatment with anti-CD80 resulted in the opposite effect (34). In contrast, CD80 appeared to be the dominant costimulatory ligand in regulating experimental autoimmune encephalitis (EAE) relapses (35). We believe that these different results may be explained, in part, by the kinetics of expression of CD80 and CD86 on individual cells in individual

organs during antigen sensitization. In this context, murine (C57BL/6) lung dendritic cells constitutively express both B7 ligands. Masten and associates have recently shown in this system that CD80 is the primary ligand needed to stimulate dendritic cell–initiated allogeneic T-cell proliferation (36). However, if dendritic cells were the essential APCs in allergic mouse lung, we would anticipate that anti-CD80 mAb treatment would have had a greater effect to inhibit airway eosinophilia and IL-4 and IL-5 secretion compared with anti-CD86 mAb treatment. Yet in our system the two individual mAb treatments had equivalent local effects *in vivo*. This suggests that multiple cell types may participate to present B7 molecules in the development of murine allergic airways. Additionally, in the current study we demonstrate by reverse transcriptase/PCR and FACS analysis that both CD80 and CD86 were expressed on lung cells from naive animals and were upregulated within 24 h of antigen challenge. We also found that both B7 molecules were expressed on eosinophils from the allergic lung and that there was greater expression of CD80 than CD86 on these inflammatory cells.

Thus, B7 expression on lung cells in this animal model of respiratory atopy does not conform to the paradigm of minimal constitutive expression and antigen-induced "early" CD86, "late" CD80 expression originally described for human B cells (37). This further suggests that expression of B7 molecules on potential APCs varies depending not only on the time they are examined and on the organ from which the cells are harvested, but also on the local environment in which the animals are kept. Specifically, eosinophils are typically not present in nonallergic tissues, therefore asthma and atopic diseases may represent a somewhat unique setting for eosinophils to function as presenting cells for costimulatory molecules. We speculate that this population of cells may act as APCs in this system to support and augment the rapidly increasing requirements for T-cell costimulation during the initial phases of an allergic response in airways. It is also likely that resident (noneosinophil) cells within the pulmonary system of "naive" animals encounter nominal antigen even though the animals have not been manipulated experimentally.

Our results are most consistent with the model that either of the B7 molecules can act as a costimulatory ligand to initiate immune reactions, but that the temporal kinetics and level of expression of either CD80 or CD86 determines the ability of either of these molecules to influence T-cell differentiation and effector functions (38). For example, Schweitzer and colleagues reported that the costimulatory signals provided by CD80 and CD86 were basically equivalent because both could elicit IL-4 as well as IFN-γ secretion by anti-CD3–stimulated CD4+ T cells, especially under suboptimal conditions (39). Similarly, *Heligmosomoides polygyrus*–infected mice seem relatively resistant to the effect of treatment with antibodies to either B7 molecule. However, treatment with antibodies to both B7 ligands could block the immune and inflammatory reactions in these animals (40). Previously, we have shown that anti-CD86 treatment exacerbates the developing stages of EAE whereas anti-CD80 treatment exacerbates already-established EAE. The different effects of anti-B7 treatment in this model are due to increased early expression of CD86 within the central nervous system followed by dominant CD80 expression later as the disease progresses (21). Thus, our results suggest that the "dominance" of either B7 molecule in "Th1" or "Th2" immune responses can be explained by a more rapid upregulation after antigen presentation (39). Our findings may also offer a temporal and functional explanation for previous studies in which CD80 or CD86 seem to play different, sometimes mutually exclusive roles in Th1/Th2 production and disease progression (11, 15, 21–24, 33, 35, 38–40).

Although treatment with anti-CD80 or anti-CD86 resulted in partial reduction in the development of allergic airways, only anti-CD86 treatment significantly depressed systemic production of IgE. These data are consistent with a previous study by Harris and associates in which CD80 blockade prevented the development of allergic airways but did not inhibit systemic production of IgE in mice (22). Treatment with anti-CD86 mAbs has previously been shown to result in deficiencies in germinal center formation and isotype switching (41). CD86- but not CD80-dependent immunoglobulin production has also been demonstrated in SV129 OVA-challenged mice (24) and the lupus-prone NZW mouse (42). Thus, while local (pulmonary) lymphocyte responses to antigen may be driven equally by CD28/CD80 or CD28/CD86 ligation, CD86 seemingly plays the predominant role in the systemic B-cell response in the mouse. This implies that constitutive expression of both CD80 and CD86 in lung may be different than in peripheral lymph node germinal centers and Peyers patches, regions that play a significant role in systemic IgE responses and where CD86 may be more dominantly expressed. We speculate that the presence of CD80-expressing eosinophils in the allergic lung but not in nonpulmonary tissues might account for these differences.

These potential regional differences in B7 expression have significant implications for developing clinical treatment strategies. Specifically, therapy for asthma that targets CD28/B7 interactions may need to consider the potential for differential expression of B7 molecules in different organs and immune environments in the same individual. This important point has recently been elegantly demonstrated in a murine model of relapsing encephalomyelitis (38). Finally, CD28/B7 inhibition after sensitization and during antigen challenge inhibited most of the allergic lung response, suggesting that targeted manipulation of the second signal involved in T-cell activation may lead to effective treatments for people with allergic respiratory disorders, including atopic asthma.

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PATIENTS AND METHODS

Four mildly allergic asthmatics with a duration of asthma of more than 2 years were included in the study after informed consent. On day 0 allergen was installed into the right lower (B7 right) and right middle lobe (B5 right) of the lung. 42 hours later a BAL was performed and bronchial biopsies were taken at the site of allergen provocation. Immediately after sampling, the BAL fluid was analyzed by flow cytometry. The expression of ICOS on bronchoalveolar T cells, identified with the CD3-specific monoclonal antibody (mAb) OKT 3, was determined by double fluorescence flow cytometry using the ICOS-specific mAb F44. The bronchial biopsies obtained from the same individuals were snap-frozen immediately after sampling. Cryostat sections obtained from the bronchial tissue were analyzed for ICOS-positive T cells by immunohistology using mAb F44 and the APAAP technique.

In addition to this study, bronchial biopsy tissue from another four allergic asthmatics were analyzed by immunohistology.

RESULTS

In normal individuals, a very low basic expression of ICOS can be observed on up to 10 - 14% of T cells in the peripheral blood using a very sensitive (phycoerythrin-based) detection system (Beier *et al.*, 2000, "Induction, binding specificity and function of human ICOS," Eur. J. Immunol. 30:3707-17, attached hereto as Exhibit A). In the four allergic individuals studied, ICOS was present on 8.3 % to 31.5% of peripheral blood T cells (FIGS. 1 and 2). This result may suggest that some allergic individuals express higher basic levels of ICOS on T cells in the peripheral blood.

In the bronchoalveolar fluid 39.4% to 69.3% of all T cells carried the ICOS antigen on the surface. In the "negative" T cell population the median of the signal was higher when compared to the peripheral blood, indicating that even most of the "negative" T cells carried low levels of ICOS (FIG. 1). In all four patients studied, the percentage of ICOS-expressing T cells in the BAL was substantially higher when compared to peripheral blood T cells of the same individuals analyzed in parallel (FIG. 2).

Bronchial biopsies taken 42 hours after segmental allergen provocation from the 4 lavaged patients were analyzed by immunohistology. In addition, bronchial biopsies taken from another 4 allergic asthmatics were examined. Characteristically, numerous ICOS positive T cells were found in the bronchial epithelium and the submucosa (FIG. 3). The proportion of ICOS positive cells found at these anatomical sites was compatible with the percentages of ICOS positive T cells found in the BAL using flow cytometry.

In the non-inflamed lung barely any T cells can be found. In the lung tissue of allergic asthmatics a substantial T cell infiltration is typically observed.

Attachments

FIG.1 *Flow cytometry profiles obtained with cells from patient 4.* In the peripheral blood, 55.4% of all cells bore the CD3 marker (and were thus identified as T cells) and 8.3% of them expressed ICOS. In the bronchoalveolar lavage the gated population contained 46.6% CD3-positive cells and 40.6% of them expressed

ICOS. Note that the numbers given in the right upper quadrants represent percentages of ICOS-positive T cells analyzed.

FIG. 2 *Percentage of ICOS-positive T cells in the peripheral blood and bronchoalveolar lavage fluid of four patients with allergic asthma 42 hours after segmental allergen provocation.*

FIG. 3 *Immunohistological staining of ICOS positive T cells* (stained in red) in a representative human bronchial biopsy. Ep=Epithelium; BM= basal membrane; SM=submucosa.

Exhibit A. Beier *et al.*, 2000, "Induction, binding specificity and function of human ICOS," Eur. J. Immunol. 30:3707-17.

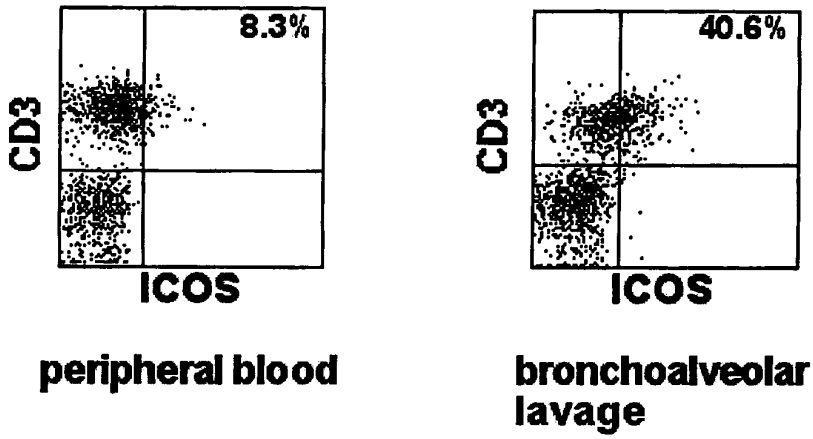
8.3%
CD3
ICOS
peripheral blood
40.6%
CD3
ICOS
bronchoalveolar lavage

FIG. 1

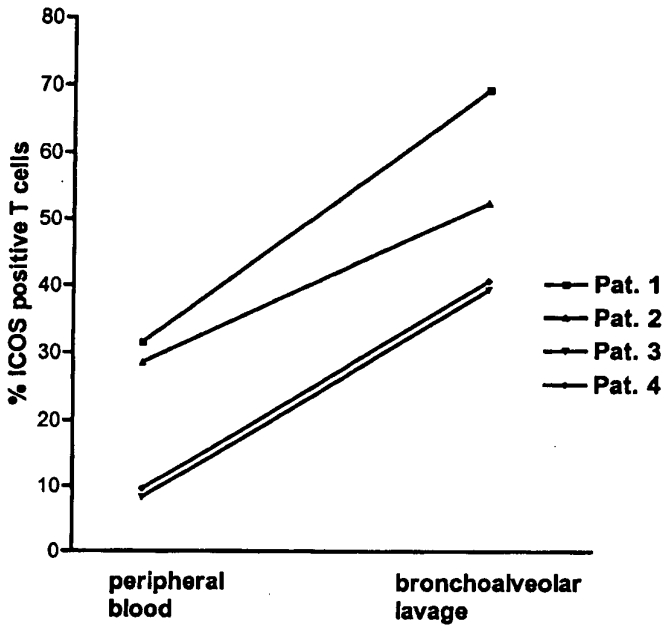
80
70
60
50
40
30
20
10
0
% ICOS positive T cells
peripheral blood
bronchoalveolar lavage
Pat. 1
Pat. 2
Pat. 3
Pat. 4

FIG. 2

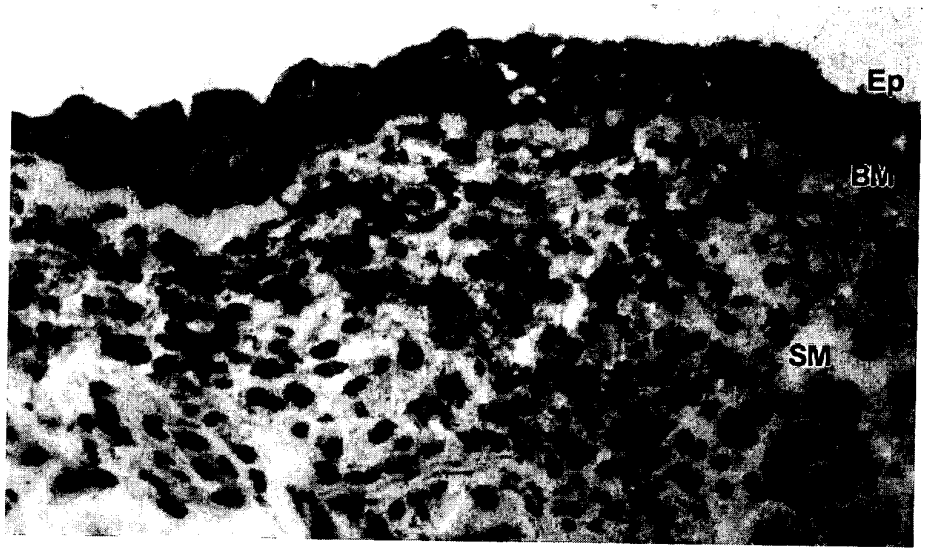

FIG. 3

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Induction, binding specificity and function of human ICOS

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Recently, we have identified the inducible co-stimulator (ICOS), an activation-dependent, T cell-specific cell surface molecule related to CD28 and CTLA-4. Detailed analysis of human ICOS presented here shows that it is a 55-60-kDa homodimer with differently N-glycosylated subunits of 27 and 29 kDa. ICOS requires both phorbol 12-myristate 13-acetate and ionomycin for full induction, and is sensitive to Cyclosporin A. ICOS is up-regulated early on all T cells, including the $CD28^-$ subset, and continues to be expressed into later phases of T cell activation. On stimulation of T cells by antigen-presenting cells, the CD28/B7, but not the CD40 ligand/CD40 pathway is critically involved in the induction of ICOS. ICOS does not bind to B7-1 or B7-2, and CD28 does not bind to ICOS ligand; thus the CD28 and ICOS pathways do not cross-interact on the cell surface. *In vivo*, ICOS is expressed in the medulla of the fetal and newborn thymus, in the T cell zones of tonsils and lymph nodes, and in the apical light zones of germinal centers (predominant expression). Functionally, ICOS co-induces a variety of cytokines including IL-4, IL-5, IL-6, IFN-γ, TNF-α, GM-CSF, but not IL-2, and superinduces IL-10. Furthermore, ICOS co-stimulation prevents the apoptosis of pre-activated T cells. The human ICOS gene maps to chromosome 2q33–34.

Key words: ICOS / CD28 / CTLA-4 / B7-1 / B7-2

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1 Introduction

The regulation of the adaptive immune system is based, among others, on a complex interaction of T cells with APC. Recognition of the peptide-MHC complex by the TCR alone is usually insufficient to fully activate the T cells and can even result in T cell anergy or apoptosis [1, 2]. An additional co-stimulatory signal is delivered by CD28 which interacts with B7-1 (CD80) and B7-2 (CD86) on APC. This co-stimulatory signal is essential for the initiation of proliferation, up-regulation of many cytokines, especially IL-2, expression of other cell surface molecules and prevention of cell death by up-regulation of anti-apoptotic genes such as bcl-x_L [1, 2]. The effects of CD28 are counterbalanced by the structurally related activation molecule CTLA-4, which also binds to B7-1 and B7-2 [2].

Recently, we identified a third member of the CD28 family of co-stimulatory molecules, which was termed "inducible co-stimulator" (ICOS) [3]. Subsequently, murine (mu) ICOS was defined by Yoshinaga et al. [4] and our group [5], and the rat homologue by Tamatani et al. [6]. Human (hu) ICOS is a disulfide-linked dimer with 39% similarity to CD28 and CTLA-4 at the protein level and is *de novo* expressed on the T cell surface following activation. In our original publication, we have principally demonstrated that huICOS co-stimulates basic T cell responses to foreign antigen, namely proliferation, secretion of lymphokines, up-regulation of molecules that mediate cell-cell interaction, and effective help for antibody secretion by B cells. The present report provides a more detailed analysis of the structure, expression and function of ICOS *in vitro*. Another focus of our work was the analysis of the structural and the functional interrelationship between the ICOS and CD28 pathways. Furthermore, an in-depth analysis of ICOS expression in human lymphoid tissues was performed.

[I 20882]

The first two authors contributed equally to this work.

Abbreviations: CsA: Cyclosporin A **DC:** Dendritic cell **FISH:** Fluorescence *in situ* hybridization **iCOS:** Inducible co-stimulator **hu:** Human **mLC:** Mature Langerhans cells **mu:** Murine **L:** Ligand

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2 Results

2.1 Chromosomal location of the hulCOS gene

Using the hulCOS cDNA of 2641 bp [3] (deposited at the EMBL database under accession no. AJ277832), a DNA probe from a genomic human library was isolated and fluorescence *in situ* hybridization (FISH) analysis performed on human metaphase chromosomes. Strong signals were obtained on chromosome 2, band q33–34, and weaker signals on chromosome 17, band q12 (Fig. 1A). Further analysis by Southern blotting of DNA from human chromosomes 2 and 17 revealed that only the signals on 2q33–34 were specific (Fig. 1B). The hulCOS gene thus is syntenic to the muICOS gene on chromosome 1 [5] and maps to the same chromosomal region as human CD28 and CTLA-4 [7, 8], suggesting that these three related genes are the result of a gene duplication event.

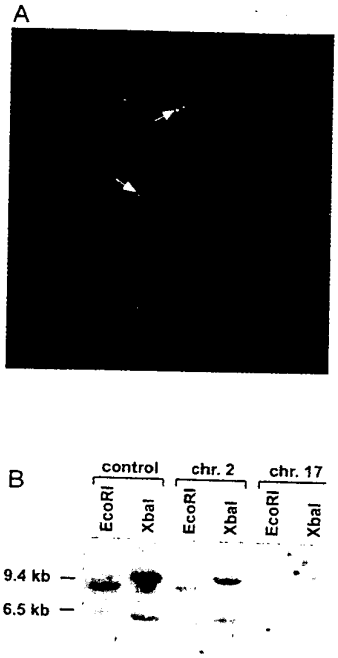

Fig. 1. Chromosomal localization of the hulCOS gene. (A) FISH analysis with a genomic hulCOS probe on human metaphase lymphocyte spreads generated strong signals on chromosome 2q33–34 and weaker signals on chromosome 17q12 (arrows). (B) Southern blot analysis of DNA from human placenta (control) and DNA from chromosomes 2 (chr. 2) and 17 (chr. 17) hybridized with the hulCOS cDNA probe revealed specific signals at approximately 9.2 kb (EcoRI digest) and approximately 6 and 10 kb (XbaI digest) only for the positive control and chromosome 2.

2.2 hulCOS is a homodimeric protein with variably glycosylated subunits

The hulCOS cDNA encodes a 199-amino acid type I transmembrane protein [3]. The cleavage site of the leader peptide has not been determined experimentally and is predicted between Thr^{19} and Gly^{20} (Y-score 0.697) or between Gly^{20} and Glu^{21} (Y-score 0.701 [9]). The protein backbone of the resulting mature ICOS protein of 179 or 180 amino acids corresponds to a M_r of 20.28 kDa or 20.22 kDa, respectively. Immunoprecipitation of ICOS with mAb F44 from activated primary human T cells revealed under non-reducing conditions a broad band between 55 and 60 kDa (Fig. 2). Under reducing conditions, two distinct bands of 27 and 29 kDa were observed, indicating that ICOS is a disulfide-linked dimer. Treatment with N-glycosidase F (ICOS protein has two potential N-glycosylation sites at Asn^{89} and Asn^{110}) resulted in a single band with an apparent M_r of 20 kDa, suggesting that the two bands at 27 and 29 kDa represent differently glycosylated variants of the same protein chain. This assumption was proven to be correct by cell surface expression of ICOS after transfecting hulCOS cDNA into L-cells (see Fig. 5). Since 20.2 kDa is the theoretical M_r of the protein backbone, additional O-glycosylation of the hulCOS protein is unlikely. Taken together, hulCOS is a homodimeric protein with two differently N-glycosylated subunits.

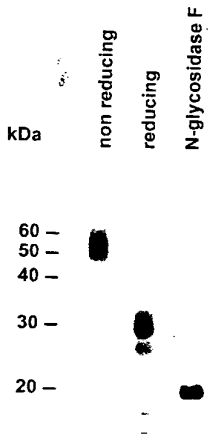

Fig. 2. Structure of hulCOS protein. T cells were activated with PMA and ionomycin for 22 h. After surface iodination of the cells, ICOS protein was immunoprecipitated with mAb F44, separated by SDS-PAGE under non-reducing or reducing conditions (12% gel) and the dried gel exposed to an X-ray film. In the right lane, the immunoprecipitate was treated with N-glycosidase F prior to reducing gel electrophoresis.

2.3 hulCOS is a "two-signal-dependent", CsA-sensitive cell surface molecule

We have earlier demonstrated the induction of hulCOS expression on the surface of $CD4^+$ and $CD8^+$ T cells using the CD3ε-specific mAb OKT3 for stimulation [3]. When $CD4^+$ T cells were activated by PMA or the calcium ionophore ionomycin instead, only a faint hulCOS expression could be observed, whereas the combined use of both agents resulted in a substantial expression of hulCOS within 8 h (Fig. 3A). The presence of cyclosporin A (CsA) during stimulation reduced the expression levels of ICOS to the levels observed with PMA alone.

2.4 Induction kinetics of hulCOS on $CD4^+$ and $CD8^+$ T cell subsets

In a next step we determined the kinetics of hulCOS expression on various T cell subsets. Interestingly, using PE-coupled mAb F44, a reagent offering highest detection sensitivity, we observed a general slight shift

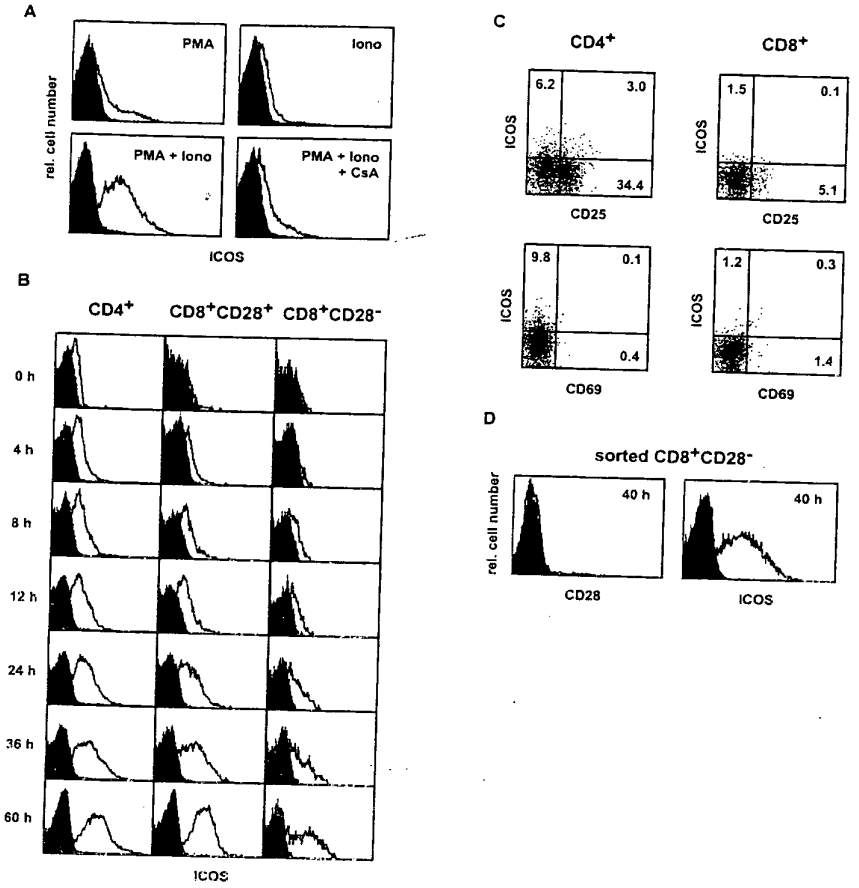

Fig. 3. Induction requirements for hulCOS. Expression of hulCOS was analyzed by flow cytometry with mAb F44 (open curve), mAb 2A11 (coupled to the appropriate chromophore) was used as an isotype control (filled curve). (A) Purified $CD4^+$ T cells were stimulated with PMA or ionomycin or with both agents for 8 h; in some experiments 1 μg/ml CsA was added to the culture prior to stimulation. Staining was performed with FITC-conjugated F44. (B) Purified $CD4^+$ and $CD8^+$ T cells, obtained from the same donor, were optimally stimulated by solid phase-bound mAb OKT3 for the indicated time periods and stained with PE-conjugated F44. The $CD28^+$ and $CD28^-$ populations were defined by electronic gating after staining with mAb CD28.2. (C) $CD4^+$ and $CD8^+$ peripheral blood cells (defined by electronic gating) were analyzed for co-expression of ICOS (PE-conjugated mAb F44) and the activation markers CD25 and CD69 (percentage of positive cells shown as inserted numbers). (D) Highly purified $CD8^+CD28^-$ peripheral blood T cells were optimally stimulated by solid phase-bound mAb OKT3 for 40 h and stained with PE-conjugated mAb F44. All experiments were at least performed twice.

towards ICOS positivity with the CD4+ peripheral blood T cell population, with 5–10% of cells reaching fluorescence levels above a threshold defined by isotype and cold blocking controls, whereas the CD8+ T cells were completely negative (Fig. 3B and C). These ICOS-expressing CD4+ T cells, not detected earlier using mAb F44 coupled to FITC [3], were CD69−, negatively correlated to the expression of CD25 (Fig. 3C), but positively correlated to the expression of CD45RO (not shown), and may thus represent T cells at a late stage of activation. When purified CD4+ (virtually all CD28+) and purified CD8+ T cells were stimulated with solid phase-bound mAb OKT3, a *de novo* induction of ICOS on the cell surface of CD4+ and CD8+CD28+ could be observed after 4 h, and the expression of ICOS continued to increase for up to 60 h (Fig. 3B). Of special interest was a subpopulation of CD8+ T cells which no longer expresses CD28. This subpopulation encompasses 10–30% of human CD8+ T cells in healthy individuals, but may represent a very high proportion of CD8+ T cells in AIDS, rheumatoid arthritis or systemic lupus erythematosus [10, 11]. When these CD8+CD28− T cells (defined by electronic gating) were activated via CD3, ICOS again could be induced onto the cell surface (albeit with slower kinetics, Fig. 3B). The activation of highly purified CD8+CD28− cells by solid phase-bound mAb OKT3 confirmed this observation (Fig. 3D) and thus demonstrated that the induction of ICOS is not absolutely dependent on the presence of CD28 on the cell surface.

2.5 The CD28/B7 pathway but not the CD40L/CD40 pathway is involved in huICOS up-regulation following antigen recognition

The observation that CD28− cells express ICOS more slowly and to a lower degree prompted us to investigate the involvement of CD28 in the induction of ICOS in more detail. To this end we used cell systems in which T cell activation occurs as a result of a complex cell-cell interaction resembling physiological antigen recognition. CD4+ T cells were activated in an MLR with allogeneic tonsillar B cells over 6 days or by superantigens presented by mature Langerhans cells (mLC) [12] for 12 h. In both systems, ICOS was potently induced onto the cell surface (Fig. 4A). Neither the presence of CD40-specific blocking mAb 89 (or mAb G28-5) nor the presence of CD80-specific blocking mAb L307.4 during the entire culture period affected the induction of ICOS (Fig. 4A). However, CD86-specific blocking mAb IT2.2 very significantly diminished the induction of ICOS onto the T cell surface and this effect was further pronounced through the combined use of mAb IT2.2 and L307.4 (Fig. 4A). These experiments clearly determined that in the course of T cell activation following antigen recognition the CD28/B7 pathway, but not the CD40 ligand (L)/CD40 pathway, is involved in the induction of the ICOS molecule onto the cell surface. Further indirect support for this conclusion came from experiments demonstrating that co-stimulation of T cells via CD28 very substantially increases ICOS cell surface expression when compared to T cell activation through CD3 alone (Fig. 4B).

2.6 huICOS and huICOS-L do not cross-intereact with the CD28/B7 pathway

The lack of a fully conserved MYPPPY motif in the huICOS protein sequence [3], which is required in its intact form for the binding of CD28 and CTLA-4 to B7-1

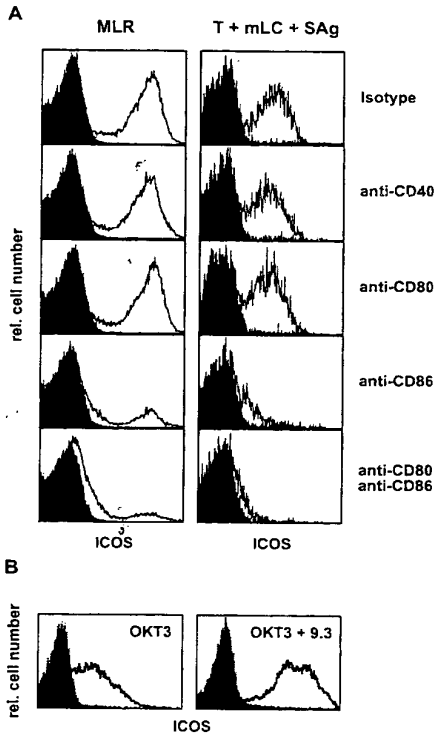

Fig. 4. Involvement of the CD28/B7 pathway in the induction of ICOS following antigen recognition. (A) CD4+ T cells were co-cultured in an MLR with allogeneic tonsillar B cells for 6 days or co-cultured with mLC in the presence of a mixture of superantigens (SAg) for 12 h, either in the presence of an isotype control mAb, or in the presence of blocking mAb directed to CD40, CD80 or CD86. Expression levels of huICOS were determined with PE-conjugated mAb F44. (B) CD4+ T cells were activated through CD3 using mAb OKT3 or co-stimulated via CD28 using a combination of mAb OKT3 and mAb 9.3 for 24 h. The expression levels of ICOS were determined by flow cytometry using PE-conjugated mAb F44. In all experiments mAb 2A11 was used as the isotype control. Shown are representative experiments out of three.

(CD80) and B7-2 (CD86) [13, 14], suggested that huICOS binds to a different counter-receptor. Recently, a new murine B7-related molecule, B7h, has been cloned [15] and we could demonstrate that B7h is the murine ICOS ligand (muICOS-L) [5]. The same central finding was obtained by Yoshinaga et al. [4], and by Ling et al. [16], two groups which also independently identified the muICOS-L as B7RP-1 and mGL-50, respectively. Subsequently, we re-cloned huICOS-L cDNA (corresponding to human GL-50 [16]), generated a soluble huICOS-L-Ig chimeric fusion protein and analyzed whether the human CD28 and ICOS pathways cross-interact. L-cells stably transfected with human CD28 bound, as expected, multimerized soluble human B7.1-Ig and B7.2-Ig, but did not significantly interact with huICOS-L-Ig (Fig. 5). On the other hand, L-cells transfected with huICOS completely failed to bind B7.1-Ig and B7.2-Ig, but specifically bound huICOS-L-Ig (Fig. 5). This experiment determined that the CD28/B7 and the ICOS/ICOS-L pathways do not cross-interact on the cell surface.

2.7 Expression of huICOS *in vivo*

Our original work demonstrated that huICOS is specifically expressed on T cells [3]. We therefore performed an in-depth analysis of ICOS expression in lymphoid organs. In eight examined fetal thymi (80–98 days of gestation) we found substantial numbers of ICOS$^+$ cells in the medulla and fewer positive cells scattered in the cortex (Fig. 6A), and a similar pattern was seen in a thymus of a 14-day-old newborn (Fig. 6B), suggesting a participation of ICOS in the early ontogeny of T cells. These results differ from our data obtained with thymic tissue of elderly individuals obtained at autopsy, where only very few ICOS$^+$ cells can be observed [3].

We have previously shown that ICOS is predominantly expressed in the apical light zone of germinal centers of human tonsils and other lymphoid tissues [3]. Typically, the number of ICOS$^+$ T cells in the T cell zones of lymphoid tissues is relatively low. Occasionally, however, a substantial proportion of cells in the T cell zones can be found to be ICOS positive (Fig. 6C). These results indicate that ICOS has not only a function in the final maturation of B cells in the germinal center, but also participates in certain phases of antigen recognition by T cells in zones containing high numbers of dendritic cells (DC). This conclusion, drawn from histological analysis, is supported by induction of ICOS on CD4$^+$ T cells after antigen recognition on mLC (compare Fig. 4) and monocyte-derived DC (own unpublished data) and by the observed expression of the ICOS-L on human B cells and DC (own unpublished data).

2.8 Co-stimulation of cytokine synthesis by huICOS alone or in combination with CD28

We have previously shown that huICOS exhibits a strong co-stimulatory activity on various parameters of T cell activation such as proliferation, expression of other cell surface activation antigens, cytokine synthesis (IL-4, IL-10) and T cell help for B cells [3]. We have now extended the analysis of the cytokine pattern induced by huICOS to more completely assess the biological effects of ICOS-mediated signals. CD4$^+$ T cells were optimally triggered via CD3 alone using mAb OKT3 together with an isotype control mAb, or using a combination of OKT3 and huICOS-specific mAb F44. Co-stimulation with mAb 9.3, one of the most potent CD28-specific reagents, was performed in parallel for comparison. With the exception of IL-6, which was less co-stimulation dependent, only small amounts of cytokines were produced at best when T cells were stimulated via the TCR complex alone (Fig. 7A). When co-stimulating T cells via ICOS, the levels of secreted IL-4, IL-5, IL-6, IFN-γ, TNF-α and GM-CSF reached between 30% and 90% of the cytokine lev-

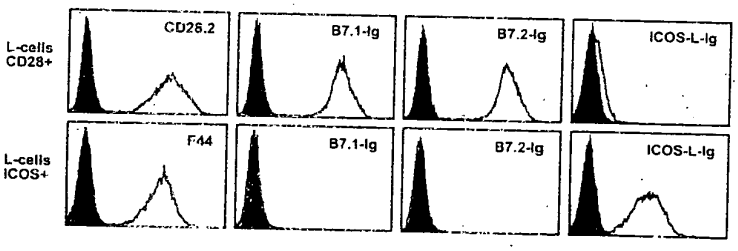

Fig. 5. huICOS and huICOS-L do not cross-interact with the CD28/B7 pathway. L-cells transfected with CD28 or with huICOS were stained with mAb CD28.2 and mAb F44, respectively, or with the chimeric proteins B7.1-Ig, B7.2-Ig or ICOS-L-Ig, and analyzed by flow cytometry (open curves). The black curves represent the signal obtained by staining untransfected L-cells with the respective reagents. Shown is one representative experiment out of two.

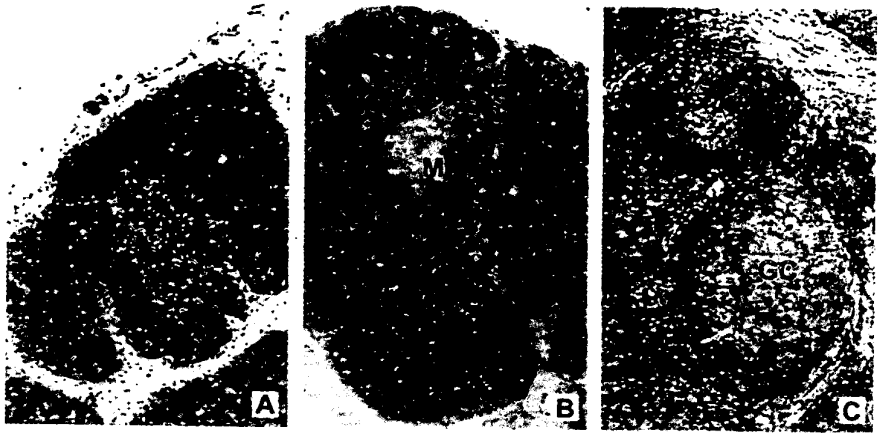

Fig. 6. Immunohistological analysis of ICOS expression in lymphoid tissue. Frozen sections of (A) a fetal thymus (91 days) and (B) an infant thymus (2 weeks) and (C) a tonsil were stained with mAb F44. M = medulla, C = cortex, GC = germinal center, TZ = T cell zone.

els obtained with CD28 co-stimulation (Fig. 7A). As reported earlier [3], there were two important exceptions: only CD28 co-stimulation led to the expression of IL-2, whereas co-stimulation via ICOS was clearly more effective in the induction of IL-10 (range: 1.5-fold to 20-fold). Interpreting these data, one has to consider that only approximately 50% of the T cells express significant levels of ICOS under these experimental conditions [3], whereas almost all of the cells are $CD28^+$. Thus, on a per cell basis, ICOS matches the co-stimulatory effect of CD28 for most cytokines and is clearly superior in the induction of IL-10. To confirm these results also on the mRNA level, we performed RNAse protection assays for some of the cytokines and obtained results consistent with the secretion profiles at the protein level (Fig. 7B). In addition, the RNA protection assay suggested that ICOS co-stimulation may be also more effective than CD28 co-stimulation for the induction of IL-9 (Fig. 7B), for which no commercial ELISA is available at present.

Since CD28 and ICOS are co-expressed by a high proportion of T cells *in vivo*, we also addressed the effect of a combined co-stimulation via ICOS and CD28. To this end, T cells were activated via CD3 as described above, co-stimulated by the simultaneous addition of mAb 9.3 and F44, and the cytokine levels were determined by ELISA. As shown in Fig. 7C, ICOS clearly did not exert any dominant negative effects on CD28-induced IL-2 secretion. For IL-10 secretion, ICOS co-stimulation only slightly increased the CD28-mediated signal (Fig. 7C) and similar minor effects could be observed with other cytokines (data not shown).

2.9 ICOS co-stimulation inhibits activation-induced T cell apoptosis

Another important function of co-stimulation is the inhibition of T cell apoptosis. It has been shown in different *in vitro* systems that suboptimal restimulation of pre-activated T cells results in apoptosis [17, 18]. Co-stimulation via CD28 can prevent this activation-induced cell death [19]. Therefore, we tested whether ICOS exhibits similar anti-apoptotic effects. T cells were activated with PHA for 6 days before restimulation. Restimulation with solid phase-bound mAb OKT3 alone resulted in massive T cell apoptosis, especially at suboptimal concentrations, whereas co-stimulation with anti-ICOS mAb significantly improved the survival rate of these T cells (Fig. 8).

3 Discussion

In the present study we have performed a detailed structural analysis of huICOS and determined the location of the huICOS gene on chromosome 2q33–34. One focus of our experiments was the analysis of the induction and expression characteristics of huICOS to obtain information shedding light on the function of this molecule *in vivo*. Another focus were experiments designed to elucidate the relationship between the structurally and functionally similar CD28/B7 and ICOS/ICOS-L pathways.

Potent induction of ICOS by PMA and ionomycin but only minimal induction by either agent alone defined huICOS as one of the few "two-signal" molecules,

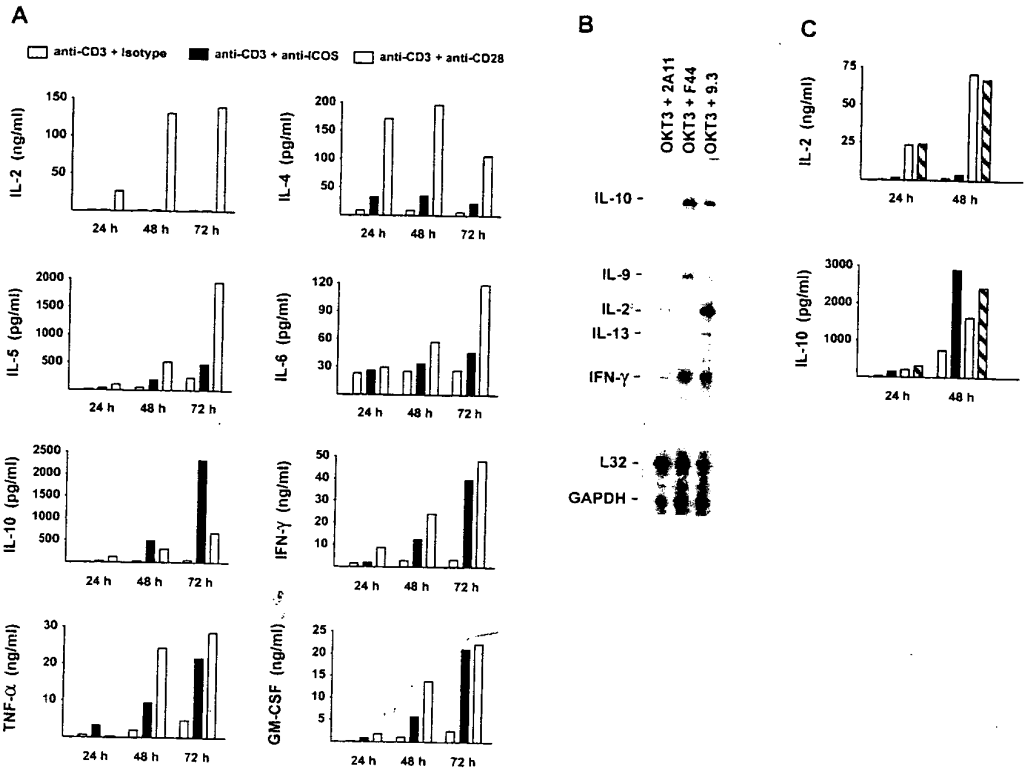

Fig. 7. Co-stimulatory effects of ICOS alone or in combination with CD28 on cytokine synthesis. (A) $CD4^+$ T cells were stimulated by optimal CD3-cross-linking (1:1,000 dilution of OKT3 ascites) in the presence of isotype control mAb MOPC-21 or mAb 2A11 (grey bars), or in the presence of ICOS-specific mAb F44 (black bars) or CD28-specific mAb 9.3 (white bars). Cell culture supernatants were assayed for cytokines after 24 h, 48 h and 72 h using commercial ELISA systems. One representative experiment out of six is shown. (B) $CD4^+$ T cells were stimulated as above (lane 1: OKT3+2A11, lane 2: OKT3+F44, lane 3: OKT3+9.3). After 20 h of culture, RNA was isolated and used for RNAse protection assays. (C) $CD4^+$ T cells were stimulated as above. In addition to the co-stimulation via ICOS or CD28 alone, the cells were co-stimulated via both cell surface antigens using a combination of mAb F44 and mAb 9.3 (hatched bars). Cytokines present in the supernatants were assayed by ELISA at the indicated time points.

among them IL-2, PILOT/EGR-3 and CD40L [20], which seem to play critical roles in the immune response. The immunosuppressive agent CsA targets all of these molecules and also suppresses the expression of hulCOS (Fig. 3A). The fast and potent activation by PMA and ionomycin also revealed expression of ICOS on virtually all peripheral blood T cells (data not shown). This finding suggests that the biological role of ICOS in the immune system is relatively broad. A number of observations support this notion. First of all, substantial expression of ICOS in the medulla of the fetal and young human thymus suggests that ICOS is involved in the early T cell ontogeny. The predominant expression of ICOS in the apical light zone of germinal centers in tonsillar and lymph node tissue ([3] and results not shown) indicates a significant role of ICOS in the T cell help for B cells. The data presented in this report also demonstrate a less pronounced but significant expression of ICOS in the T cell zones of lymphoid tissues, which suggests an involvement of ICOS in the recognition of antigen presented by the DC at this site, and this assumption is supported by the observation of ICOS-L expression on DC *in vitro* (own unpublished data). Finally, the induction of ICOS on virtually all $CD8^+$ T cells, including the $CD28^-$ subset, suggests that ICOS also participates in the immune defense against intracellular bacteria and viruses.

The analysis of the expression kinetics of ICOS on T cells activated via CD3, in some experiments in direct comparison to the expression of CD69, CD40L or CD25 (data not shown), revealed that hulCOS is induced early (but later than CD69 and CD40L), and remains on the cell surface for extended periods of time. Combined with the strong positive correlation of ICOS with $CD45RO^+$ *in vivo*

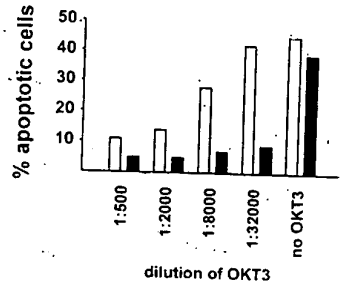

Fig. 8. Co-stimulation via ICOS rescues activated T cells from apoptosis. T cells were stimulated for 6 days as described in Sect. 4.10, and re-stimulated with solid phase-bound mAb against CD3 (mAb OKT3, 1:500–1:32,000 dilution of ascites) in the presence of the isotype control mAb 2A11 (grey bars), or in the presence of anti-ICOS mAb F44 (black bars) for 24 h. The percentage of apoptotic cells was determined by flow cytometry after propidium iodide staining of nuclear DNA.

[3], these findings suggest that ICOS is involved both in the early and the late phases of an immune reaction.

The functional experiments performed earlier [3] and in this report demonstrate that CD28 and huICOS resemble each other in many respects. Both molecules potently co-stimulate T cell proliferation, expression of activation antigens and the synthesis of a variety of cytokines, and are thus able to provide T cell help for B cells. Similar to CD28 [19], ICOS is able to prevent apoptosis of pre-activated T cells. Nevertheless, these experiments revealed several significant differences between CD28 and huICOS. First of all, co-stimulation via huICOS does not lead to the production of significant amounts of IL-2, as is observed with CD28. Secondly, the relative efficiency in the co-stimulation of various cytokines somewhat differs between the two molecules, with the most pronounced difference observed for IL-10 (see Fig. 7A), and this may have major implications for the biological roles of CD28 and ICOS *in vivo*.

Given the overall structural and functional similarity of CD28 and huICOS, we performed a series of experiments aimed to determine the inter-dependence of the CD28 and ICOS pathways. The induction of ICOS on $CD8^+CD28^-$ human T cells following OKT3 stimulation (Fig. 3D) suggested that CD28 is not absolutely required for the expression of huICOS and this interpretation is congruent with the finding that T cells from CD28 knock-out mice express ICOS upon activation [4]. However, in cell culture systems where T cell activation results from a complex interaction with APC, blockade of the CD28/B7 pathway drastically reduced the induction of ICOS onto the T cell surface, whereas the blockade of the CD40L/CD40 pathway remained without effects. These experiments indicated that CD28 is critically involved in the induction of huICOS following antigen *in vivo*.

Using transfectants, we determined that soluble B7–1 and B7–2 do not bind to huICOS. At the same time, we observed that soluble ICOS-L does not show any significant reaction with CD28. These staining experiments indicate thus that there is no significant cross-interaction between the CD28 and ICOS pathways on the cell surface.

Taken together, our data suggest a hierarchy of T cell co-stimulation *in vivo*. Upon initial interaction of T cells with APC, CD28 interaction with the B7 molecules induces ICOS onto the T cell surface. Subsequently, CTLA-4 is up-regulated and terminates the interaction of CD28 with B7–1 and B7–2 [2]. In this second phase of cellular cross-talk, ICOS could continue to co-stimulate T cells, and by modifying the pattern of T cell cytokines secreted, initiate the next stage of the immune response. The observed overproduction of IL-10 following huICOS signaling could have different sequelae, depending on the type and location of APC interacting with the T cells. In the T cell zone, the increased secretion of IL-10 could down-regulate the antigen-presenting ability of DC and promote their apoptosis [21]. In the germinal center, on the other hand, the IL-10 could lead to the terminal differentiation of B cells into plasma cells in the apical part of the light zone [22–24], the major site of ICOS expression [3]. The latter hypothesis is supported by the observation of a massive expansion of plasma cells in mice transgenic for the muICOS-L [4]. In the hierarchy model outlined here, the biological effects of CD28 and huICOS would be mostly dissociated in time, thus giving a biological meaning to the largely overlapping functional effects of CD28 and huICOS.

4 Materials and methods

4.1 Mapping of the huICOS gene

A genomic clone for ICOS was isolated from a human placental DNA library in lambda FixII (Stratagene) using huICOS cDNA as a probe. For FISH, metaphase lymphocyte spreads from a healthy individual were pretreated for 3 min with 50 ng/ml RNAse and for 10 min with 0.05% pepsin. The genomic huICOS DNA clone was biotinylated, hybridized overnight to the metaphase spreads and the signals generated using FITC-avidin/anti-avidin [25]. For Southern blot analysis, genomic DNA from human placenta, chromosome 2 (human/hamster hybrid cell line NA10826B) or chromosome 17 (human/mouse hybrid cell line NA10498, both from Coriell Institute for Medical Research, Camden, NJ) was

digested with EcoRI or XbaI and analyzed by standard procedures.

4.2 Immunoprecipitation

Nylon wool-purified human T cells (40×10^6) were activated for 22 h with 20 ng/ml PMA and 200 ng/ml ionomycin, surface iodinated with 0.3 mCi ^{125}I using IODO-Beads (Pierce), lysed in a NP40 buffer and immunoprecipitated with mAb F44 coupled to protein G-Sepharose as described [3]. Immunoprecipitates were separated by reducing or non-reducing SDS-PAGE (12% gel). To determine the molecular mass of the polypeptide backbone of ICOS, immunoprecipitates were treated with 4 U N-glycosidase F (Roche Biochemicals) prior to separation. After electrophoresis, gels were dried and exposed to an X-ray film.

4.3 Purification of cells

Peripheral blood $CD4^+$ and $CD8^+$ T cells were negatively purified from buffy coats using nylon wool adherence and magnetobead-coupled (Dynal) mAb against CD19, MHC-II, CD11b and CD8 (for $CD4^+$) or mAb against CD19, MHC-II, CD4 and CD16 (for $CD8^+$). The purity achieved was always greater than 96% ($CD4^+$) or 92% ($CD8^+$), respectively. $CD8^+CD28^-$ T cells were negatively purified from $CD4^-$ cells using anti-mouse Ig beads (MACS, Miltenyi, Bergisch-Gladbach) and mAb CD28.2 (a kind gift of D. Olive) and contained virtually no $CD4^+$, $CD28^+$, $CD19^+$, $CD11b^+$, $CD16^+$ or MHC-II^+ cells. Tonsillar lymphocytes were isolated from mechanically dispersed tonsils obtained from routine tonsillectomies (from patients aged 3–18 years) using Ficoll-Hypaque gradient centrifugation. Tonsillar B cells were further purified by rosetting the T cells with 2-aminoethyl isothiourea-treated SRBC and were routinely 98% pure. mLC were isolated as described earlier [12].

4.4 Stimulation of cells

T cells were stimulated with 1.5 μg/ml PHA at 0.5×10^6/ml, or with 33 ng/ml PMA or 200 ng/ml ionomycin at 2×10^6/ml. CsA (Novartis) was dissolved in 100% ethanol and used at a final concentration of 1 μg/ml. For all stimulations with mAb, T cells were cultured in 96-well round-bottom microtiter plates pre-coated with 10 μg/ml rabbit anti-mouse Ig (Sigma) followed by mAb against CD3 (OKT3, ATCC; 1:1,000 dilution of ascites, if not otherwise indicated), ICOS (mAb F44 [3]; 4 μg/ml), or CD28 (mAb 9.3 [26], kind gift of J. Ledbetter; 1:3,000 dilution of ascites). mAb 2A11 (own hybridoma) or MOPC-21 (Sigma), both at 4 μg/ml, were used as isotype controls. Cytokines in the cell culture supernatants were determined using commercial ELISA (Biosource). For the MLR 25,000 $CD4^+$ T cells were co-cultured with 50,000 allogeneic tonsillar B cells in round-bottom 96-well microtiter plates. $CD4^+CD45RA^+$ T cells were co-cultured with mLC at a ratio of 20:1 in flat-bottom microtiter plates in the presence of the superantigens SEA, SEB, and TSST added at optimal concentrations (determined by titration experiments) of 0.1 ng/ml each (~5 pM). Incubation of T cells with the mixture of superantigens alone did not induce expression of T cell activation markers or proliferation.

4.5 Flow cytometry

Flow cytometry was performed on a FACSCalibur (Becton Dickinson), the mLC cultures were analyzed on a Coulter Profile II. The following mAb (coupled to FITC, PE, Cy-Chrome or Cy5) were used: HIT8a (anti-CD8), HIB19 (anti-CD19), CD28.2 (anti-CD28), L307.4 (anti-CD80), FN50 (anti-CD69), IT2.2 (anti-CD86, all Pharmingen), F44 (anti-ICOS, [3]), 2A3A1H (anti-CD25, ATCC). mAb 2A11 (IgG1, own hybridoma), coupled to the appropriate chromophore, was used as an isotype control in all experiments. In all analyses, even after purification, $CD8^+$ T cells were electronically gated using mAb HIT8a-CyChr. B cells and DC were pre-incubated before staining with mAb with Endobulin (human IgG, Immuno, Heidelberg) to block FcR. The secondary reagent for ICOS-L-Ig was PE-conjugated goat anti-rabbit-Ig (Southern Biotechnology), for B7.1-Ig and B7.2-Ig it was FITC-conjugated goat anti-human $IgGF_c$ (Jackson).

4.6 Transfectants and cell lines

cDNA for ICOS, CD28 and CD40L were cloned into the expression plasmid BCMGSneo [27] and stably transfected into mouse fibroblastic Ltk^- cells (L-cells; kindly provided by J. Banchereau) by electroporation.

4.7 Ig fusion proteins

A BlastX search with sequence of the murine ICOS-L [15] revealed a human cDNA clone (accession no. AB014553) encoding a polypeptide with striking homology (46.6% amino acid identity within the extracellular domain). Based on the nucleotide sequence of AB014553 the extracellular domain of the huICOS-L was amplified by standard reverse transcription (RT)-PCR using RNA obtained from PBMC and the oligonucleotide primers 5'TTAAGAATTCCAC-CATGCGGCTGGG 3' (forward, the underlined sequence corresponds to nucleotides 125–139 of AB014553) and 5'TTAAGGATCCCTCTCCGATGTCATTTCCTGTC 3' (reverse, the underlined sequence corresponds to nucleotides 842–821 of AB014553). The resulting PCR product was cloned into the *Drosophila* expression vector pRmHa-3 in frame to the Fc-region of rabbit IgG by standard cloning procedures. Transfection of Schneider SL-3 cells with the recombinant plasmid as well as production and purification of the soluble fusion protein was performed as described earlier [5]. B7.1/B7.2-Ig proteins (CD80/86-Igαtp) have the tailpiece of human IgA at the end of the human IgG

sequence which leads to formation of a hexameric Ig fusion protein, and were a kind gift of R. Sweet (SmithKline Beecham Pharmaceuticals).

4.8 Immunohistology

The infant thymus was obtained from the Deutsches Herzzentrum, Berlin, and fetal thymi (80-98 days) from Department of Pediatrics, University of Washington, Seattle. Cryostat sections were incubated with mAb F44 followed by biotinylated goat anti-mouse antibody. The color reaction was developed either by using streptavidin-horseradish peroxidase (HRP) and 3-amino-9-ethylcarbazole as substrate or with ABComplex/HRP (Dako) and 3,3'-diaminobenzidine for the fetal thymus. Nuclei were counterstained with hematoxylin.

4.9 RNAse protection assay

$CD4^+$ T cells (0.5×10^6/ml) were stimulated with mAb as described in Sect. 4.4 for 20 h and the RNA extracted with TRIZOL (Gibco). The RNAse protection assay of a template set of cytokines (Pharmingen) was performed according to the manufacturer's instructions.

4.10 Apoptosis assay

T cells (2×10^6/ml) were stimulated with 1 μg/ml PHA for 20 h, washed, and cultured for additional 5 days in IL-2-containing medium (25 U/ml). Dead cells were then removed by Ficoll-Hypaque gradient centrifugation and restimulated with various dilutions of mAb OKT3 ascites for 24 h as described in Sect. 4.4. Thereafter, the cells were fixed in ethanol for 18 h, treated with RNAse, stained with propidium iodide, and analyzed by flow cytometry for fragmented DNA [28].

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Note added in proof: During review of this manuscript we became aware of a publication by Brodie et al. (*Curr. Biol.* 2000 **10:** 333) which also shows that soluble ICOS-L does not bind to CD28.